The Role of Neural Crest Cells in the Development, Organisation and Migration of the Thymus

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Declaration:

I, Katie Foster confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Neural Crest (NC) derived mesenchyme has previously been shown to play an important role in the early development of the foetal thymus. Using Wnt1-Cre and Sox10-Cre mice crossed to Rosa26^{eYfp} reporter mice, NC derived mesenchymal cells were revealed in the adult murine thymus. It is reported here that NC derived cells infiltrate the thymus before E13.5, and differentiate into cells with characteristics of smooth muscle cells associated with large vessels, and pericytes associated with capillaries. In the adult organ at three months of age, NC derived perivascular cells continue to be associated with the vasculature providing structural support to the blood vessels and possibly regulating endothelial cell function.

Thymus organogenesis requires co-ordinated interactions of multiple cell types including NC cells that orchestrate the formation, separation and subsequent migration of the developing thymus from the third pharyngeal pouch to the thoracic cavity. The molecular mechanisms driving these processes are unclear, however NC derived mesenchyme has been shown to be important. Here, it is shown that the separation process of the thymus from the pouch is independent of ephrin-B2 expression on thymic NC derived mesenchyme, however in its absence the thymus remains in the cervical area instead of migrating into the thoracic cavity. Analyses of individual NC derived thymic mesenchymal cells shows that the absence of

ephrin-B2 impairs their polarisation, and thus motility, as a result of defective EphB receptor signalling. This implies a NC derived cell specific role of EphB-ephrin-B2 interactions in the collective migration of the thymic rudiment during organogenesis.

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Figures and Tables

Abbreviations

%	Percentage
AB IMDM	Air buffered Iscove's Modified Dulbecco's Medium
AOBS	Acousto-Optical Beam Splitter
AP	Alkaline Phosphatase
APC	Allophycocyanin
BABB	Benzyl Alcohol : Benzyl Benzoate
BCIP	5-Bromo, 4-Chloro, 3' Indolyphosphate p-Toludine salt
bp	base pair
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CY3	Cyanine 3
ddH_2O	double distilled water
DIG	Digoxygenin
DMEM	Dulbecco's Modified Essential Medium
DNA	Deoxyribonucleic Acid
dNTP	deoxyribonucleotide
E9.5-18.5	Embryonic day of development
EDTA	Ethylene Diamine Tetracetic Acid
eYFP	enhanced Yellow Fluorescent Protein
FACS	Fluorescence Activated Cell Sorting

FCS	Foetal Calf Serum
GFP	Green Fluorescent Protein
Ig	Immunoglobulin
MHC	Major Histocompatibility Complex
NBT	Nitro-blue Tetrazolium Chloride
NC	Neural Crest
NCC	Neural Crest Cell
nm	nanometer
NTMT	NaCl, Tris-HCl, $MgCl_2$, Tween 20
OCT	Optimal Cutting Temperature
PBS	Phosphate Buffered Saline
PBT	Phosphate Buffered saline with Triton-X 100
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PE	Phycoerythrin
PFA	Paraformaldehyde
PK	Proteinase K
rpm	rotations per minute
RPMI	Roswell Park Memorial Institute 1640 Medium
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
SMA	Smooth Muscle Actin
SSC	Sodium chloride, Sodium Citrate

TAE	Tris Acetate EDTA
TBST	Tris Buffered Saline with Tween 20
UTP	Uridine Triphosphate
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
w/v	weight per volume

Publications

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Chapter One: Introduction

1.1 Overview of the immune system

In order for an organism to survive, it must be able to defend itself from infection by disease-causing pathogens and even basic immune responses are present in unicellular bacteria. A number of mechanisms have evolved to recognise and eliminate pathogens. Higher vertebrates have developed complex adaptive defence mechanisms consisting of specialised cells, organs and tissues, which interact through cell contact and secreted molecules (1). The adaptive immune response in vertebrates is specific to each different pathogen encountered and allows each recognised antigen to be remembered. This immunological memory provides for a faster and stronger response to secondary encounters with the same pathogen.

The cells of the adaptive immune response, B and T cells, are derived from hematopoietic stem cells generated in the bone marrow and foetal liver. B cells develop in the bone marrow and are involved in humoral responses. In contrast, T cells develop in the thymus and are involved in cell-mediated immune responses. T cells recognise a peptide-antigen target, such as a pathogen, through T cell receptor (TCR) binding to peptide antigen processed and presented in combination with a “self” Major Histocompatibility Complex (MHC) molecule. There are two major TCR bearing T cell classes; αβTCR and γδTCR. αβT cells can be subdivided into two major types; cytotoxic, which express the CD8 cluster of differentiation

gene and recognise antigen coupled to Class I MHC molecules; and helper, which express the CD4 gene and recognise Class II MHC molecules. γδ T cells can recognise intact antigen not bound to MHC receptors (2).

The thymus is the site of T cell maturation and selection. Immature T cell progenitors generated in the bone marrow enter the circulating blood supply, and home to the thymus. In the thymus, they undergo differentiation into mature naïve T cells that are exported into the periphery. The thymus has a distinct structure consisting of epithelial cells (TECs), mesenchymal cells, thymic macrophages, thymic dendritic cells (DCs), developing T cells and blood vasculature, generating a unique microenvironment. Through interactions with thymic epithelial and dendritic cells, immature thymocytes undergo positive and negative selection. Only those T cells expressing non-self antigen with the correct affinity and avidity for MHC molecule-peptide conjugates will survive and be exported to the periphery. In contrast, those which react at high affinity with self, or do not receive a positive MHC-TCR interaction, will be eliminated.

1.2 Thymus development

The thymus is formed from cells in the pharynx, a widened anterior portion of the foregut lined with a layer of endodermal cells, which expands into the pharyngeal pouches extending between the pharyngeal arches (Figure 1.1A). Development of the thymus in the mouse begins at E9.5 and involves the expression of transcription factors including Hoxa3, Pax1/9, Eya1 and Six1 (3-9) within the third pharyngeal pouch endoderm and neural crest (NC) derived mesenchyme (Figure 1.1B). Epithelial cells derived from the endoderm begin to proliferate causing the pouch to grow and bud to form a shared thymus and parathyroid primordium. Simultaneously, NC derived mesenchymal cells derived from the anterior to fifth somite in the posterior hindbrain undergo an epithelial to mesenchymal transition (EMT), delaminate from the neural tube and migrate towards the bilateral epithelial pouches, completely surrounding them (Figure 1.1C). Subsequently, the epithelial bud detaches from the pharynx and separates into two separate primordia; the thymus, which gradually descends into the thoracic cavity where it resides above the heart by E13.5; and the parathyroid, which remains in the neck and becomes associated with the thyroid. The molecular mechanisms that control separation and migration are unclear, but appear to involve, amongst others, Pax3, Pax9, Alk5, Fibroblast Growth Factors (FGFs), FGF Receptor Substrate 2α (FRS2α) and Bone Morphogenic Proteins (BMPs) (Figure 1.1D). Lymphoid progenitor cells from the aorta-

gonad-mesonephros (AGM) circulating in the nascent blood supply leave vessels nearby to the rudiment and migrate through the surrounding perithymic mesenchyme to enter the thymus primordia starting after E11.5 (10, 11), and begin the process of proliferation and differentiation (reviewed in (12)).

1.3 Initiation and Specification

The mechanisms of initiation and positioning of the thymic rudiment are unknown, but appear to involve signals emanating from the endoderm (13). The transcription factor network Hoxa-Pax-Eya-Six is considered to be a likely candidate for this process, since in their absence the third pharyngeal pouch forms normally, but the thymus and parathyroids which arise from it are often hypoplastic or missing (3-9). A similar signalling cascade involving Pax, Eya and Six operates to control development of the eye in Drosophila (14) and patterning of the kidney region in vertebrates (Reviewed in (15)). Since the anterior boundary of Hoxa3 expression is the third pharyngeal pouch, an attractive hypothesis is that the Hox-Pax-Eya-Six pathway might determine the position of initial rudiment formation. This hypothesis is based on the model of colinear expression of Hox genes in different embryonic axes, whereby translation of genetic information into a combinatorial code controls the regulation of regional identity (16).

Hoxa3 is expressed at E9.5 in the third pharyngeal pouch endoderm and NC derived mesenchyme, and appears to be a crucial player in thymus organogenesis. Mice deficient in Hoxa3 have defects in the derivatives of the third pharyngeal arch and pouch, and lack thymus and parathyroid glands. Part of the role of Hoxa3 in thymus development may be its regulation of Pax1 and Pax9 expression in the third pouch endoderm, since both are downregulated in Hoxa3$^{-/-}$ mutant mice at E10.5. Pax9 mutants exhibit an early failure in thymus organogenesis, whilst Pax1 mutant have more subtle defects. In Pax1 mutants a thymus rudiment does form, albeit severely reduced in size, and is colonised by T cell progenitors. However, these precursors fail to mature and the rudiments do not migrate into the superior mediastinum. Loss of one allele of Hoxa3 in Hoxa3$^{+/-}$ Pax1$^{-/-}$ compound mutants results in hypoplastic thymi and there is a delay in separation of the primordia from the pharynx leading to ectopic location of the organ.

Six1 is a member of the homeobox Six gene family, homologous to the *Drosophila sine oculis* (so) gene. Six1 is expressed in the pharyngeal endoderm, mesenchyme and ectoderm. Six proteins bind directly to DNA to regulate gene transcription, and interactions with various cofactors including Eya1 modulate their activity (14, Ohto, 1999 #1, Ikeda, 2002 #619). In the absence of Six1, the patterning of the third pouch into thymus and parathyroid primordia is normal but the endodermal cells fail to maintain the expression of Glial Cells Missing-2 (GCM2) and Forkhead transcription

factor-N1 (FoxN1), and, thus, the rudiments disappear by E12.5. *eyes absent* (Eya) 1, which encodes a transcription coactivator, is expressed in the pharyngeal endoderm, mesoderm and ectoderm at E9.5 (7, 17). In Eya1 deficient embryos the thymus and parathyroid primordia fail to form and this is thought to be due to a failure of patterning of the combined rudiment into thymus- and parathyroid-domains, rather than initiation.

Eya1 and Six1 are thought to act downstream of Hoxa3 since both mutants show normal levels of Hoxa3 expression by *in situ* hybridisation. However, it is unclear whether they act upstream or downstream of Pax1/Pax9. Zou and colleagues (8) report that whilst Pax1 is undetectable in Eya1;Six1 double mutants by *in situ* hybridisation, Pax9 expression was not altered in the pharyngeal endoderm in Six1 single or Eya1;Six1 double mutants. Thus Eya1-Six1 is required for the expression of Pax1, but not Pax9. However, in another report, Xu and colleagues (7) showed that both Pax1 and Pax9 expression levels in $Eya^{-/-}$ embryos were comparable to those in wild type controls.

Other molecules, which may contribute to the mechanisms of initiation and positioning of the thymus, include Tbx1, GCM2 and Ehox. Pharyngeal pouches do not form in Tbx1 homozygous mutants, and in these mutants NCCs fail to migrate. Tbx1, a gene implicated in the development of Di George's syndrome (18), which is characterised by anomalies, such as

cardiac outflow tract abnormalities, cleft palate, craniofacial abnormalities and thymic aplasia, resulting from defective pouch formation. The *Drosophila* gene *GCM2*, which encodes a transcription factor, is specifically expressed in the parathyroid gland during its organogenesis (19). GCM2 is expressed at E9.5 in the second and third pharyngeal pouches, and is further confined to a small domain of the third pouch endoderm by E10.5. It is proposed that its expression of GCM2 suppresses the thymus identity specified by the Hox-Pax-Eya-Six network and replaces it with a parathyroid fate. GCM2 expression itself also appears to be regulated by the Hox-Pax-Eya-Six network, since it is not initiated in the Hoxa3$^{-/-}$ (3) or Eya1$^{-/-}$ mice (7), and is downregulated in the Hoxa3$^{+/-}$ Pax1$^{-/-}$ compound mutants (5). Expression of Ehox, a distant member of the paired box of homeo-domain transcription factors, is expressed throughout the endoderm at E8.5 but is limited to a ventral domain in the second and third pharyngeal pouches by E9.5 (20). At E10.5 it is further restricted to a domain that is complementary to the GCM2-expressing parathyroid-fated domain, and thus may also have a role in thymus organogenesis. At 11.5, Ehox is not detected by *in situ* hybridisation, and instead is replaced by the expression of FoxN1.

1.4 Separation from the pharynx and migration

Prior to migration, the thymus must detach from the pharynx and parathyroid primordia. The Hox3 and Pax1/9 transcription factors act in a pathway that is

required for proper separation and/or migration of the developing thymic and parathyroid primordia from the pharynx, although the cell-type-specificity of these functions is poorly understood (5, 21, 22). In Pax9 mutants, separation of the rudiments from the pharynx does not occur, and is delayed in the Hoxa3$^{+/-}$Pax1$^{-/-}$ mutants. Hoxa$^{+/-}$Hoxb3$^{-/-}$Hoxd3$^{-/-}$ combined mutants have normal sized lobes, which do separate from the pharynx but do not migrate. *Splotch* embryos, which have a null allele of Pax3 and are largely deficient in NCCs, also exhibit pharyngeal pouch defects including an ectopic thymus (23, 24). Deficiency in NCCs results in delayed separation of the thymus and parathyroid from the pharynx, and the boundary between thymus and parathyroid-fated domains is abnormal, leading to ectopic thymyi. These results strongly implicate NCC migration to the third pharyngeal pouch in patterning and morphogenesis of the thymus and parathyroids (23). Interestingly, embryos which have deleted the TGF-β type-1 receptor, ALK5, in NC derived cells also have ectopically located thymi and delayed separation of the parathyroid from the thymic rudiment without histological or other differentiation defects. Moreover, this defect is not due to defective NC migration to the third pharyngeal pouch, but is thought to be due to increased apoptosis in post-migratory NCCs (25).

In addition to regulating mesenchymal-epithelial interactions in thymocyte development, FGFs are also important players in the separation of the thymus rudiment from the pharynx and its subsequent migration. FRS2α

mediates FGF signalling by providing a link between FGF receptors and intracellular signals. Disruption of the *FRS2α* gene is embryonic lethal at E7.0-7.5, but targeted disruption of the Shp2-binding site yields viable embryos until E18.5. In these embryos, there is impaired cerebral cortex and eye development, the carotid body is absent, the parathyroid hypoplastic and the thymus fails to migrate. Failure of the thymus to migrate results from incomplete separation of the mutant thymus from the pharyngeal epithelium (26). However, to date no mutants with ectopic thymi have been verified to have migration defects without additional defects in separation from the pharynx.

Interestingly, FGFs are also required for cranial NCC migration to the pharyngeal pouches. Several members of the FGF family are expressed in the cranial region, near the cranial regions, or both, which are colonised by NCCs. For example, FGF2 is localised in facial mesenchyme (27), which is the migratory and homing region of mesencephalic and prosencephalic NCCs. FGF8 expression is found in the epithelium of branchial arches, and is expressed during premigratory and migratory stages of cephalic NC development (28, 29). Moreover, FGF8 controls craniofacial development (30) and regulates gene expression in NC-derived ectomesenchymal cells (31), thus both FGF2 and 8 are candidates to control cranial NC migration to the pharyngeal arches (32).

FGF signalling also plays a crucial role in the formation of the great vessels associated with the heart. In addition to tissues and organs that derive from the pharyngeal pouches, each pharyngeal arch gives rise to an artery. The common carotid artery derives from the third pharyngeal arch and connects the aortic sac with the dorsal aorta. All pharyngeal arch arteries are formed by E10.5, but undergo a remodelling process leading to the mature aortic arch development and great vessel patterning at around E11.5 (33). T box 1 (Tbx1), mediated by FGFs, is required for the formation of the pharyngeal arch arteries. Tbx1 is not expressed by the structural components of the arteries, but instead in the surrounding pharyngeal endoderm.

Both Tbx1 and FGF mutants exhibit vessel and heart defects, and also have defects in thymus organogenesis. Moreover, since the thymus appears to follow the common carotid artery into the thorax during its migration, FGFs and/or Tbx1 expression may be attractive candidates in directing the migration of the thymus. However, since mutants for Tbx1 and FGFs exhibit an early defect in thymus initiation and patterning, the roles of these genes in thymus migration remain unknown.

For a summary of mice with thymic and parathyroid defects, see Table 1.1.

1.5 Vascularisation and influx of lymphoid progenitors

The thymic rudiment is first colonised by passively migrating lymphocyte progenitor cells from the nascent blood vessel network between E10.5 and E12.5 (34) in response to chemoattractant factors (35). Lymphocyte progenitors leave the blood in the region of the dorsal aorta and migrate through the perithymic mesenchyme to enter the epithelial-mesenchymal mass that constitutes the foetal thymic primordium (13, 34, 36). Chemokines, CCL21 & CCL25, are produced in the primordium and their receptors, CCR9 and CCR7, are expressed on T cell progenitors. These molecules play significant roles in the early vasculature-independent pathway (37-40). Interestingly, musculoaponeurotic fibrosarcoma oncogene homolog B (MafB), expressed on CD45-IA^{-} Platelet Derived Growth Factor Receptor alpha^{+} (PDGFRα) mesenchymal cells, also appears to be important in this process. Mice deficient for MafB exhibit significantly reduced T cell progenitor accumulation in the embryonic thymus, reduced expression of Wnt3 and BMP4 in mesenchymal cells and reduced CCL21 and CCL25 expression on epithelial cells (9).

After blood vasculature development, direct lymphocyte trafficking between the blood circulation and the thymus becomes the main mode of thymus colonisation. Functional vessels are formed at E14.5 in the thymus following inward branching of endothelial cells from existing blood vessels that are present nearby the thymic capsule (for review see (41)). Later in gestation, the thymic artery branches at the cortico-medullary boundary into many

arterioles dividing further into capillaries that ascend into the cortex and subsequently loop back to merge with post capillary venules (42).

It is thought that endothelial cells from vessels surrounding the thymus proliferate and migrate into the organ towards angiogenic stimuli, such as vascular endothelial growth factor (VEGF) (43). VEGF, produced by thymic epithelial cells and mesenchymal cells (44), is an endothelial cell mitogen and a permeability enhancing factor that stimulates angiogenesis in response to hypoxia, increased glucose concentration or pH (45). VEGF receptor positive endothelial cells upon binding VEGF are induced to proliferate, resulting in formation of sprouting processes or immature blood vessel structures. Recently, it was demonstrated that pericytes are an important source of VEGF and may migrate ahead of endothelial cells guiding the sprouting processes (46). Other factors thought to play a role in angiogenesis include angiopoietin1 that is produced by surrounding mesenchymal cells (47) and induces pericyte and vascular smooth muscle cell development. Endothelial cells produce PDGF-B that in turn attracts PDGFRß$^{+}$ perivascular cells to the walls of growing blood vessels (48). Perivascular cells, such as pericytes and smooth muscle cells, wrap around the vessels providing structural support and regulating endothelial cell function. In the absence of perivascular cells or PDGFRß signalling, vessel structures are abnormal and prone to haemorrhage (49). Indeed, this is a characteristic of pathological states such as oedema and diabetic

retinopathy, where vessels appear abnormal and fragile (50). Within the thymus cortex, the structure formed by the association of perivascular cells with the vessels themselves is unique among the lymphoid organs, resulting in vessels with a double-walled morphology. The putative function of this double-layer constituting a “blood-thymus barrier” is to block the entry of blood borne antigens into the areas where T cells undergo maturation (51, 52).

1.6 T cells in the thymus

Thymocytes are generated from bone marrow derived hematopoietic progenitors in the adult and from hemangioblasts in the embryo. Thymocytes enter the post-natal thymus in waves, a process dependent upon the maturation state and number of cells already within the organ (13, 53, 54). Once inside the thymus, progenitors undergo complex selection and maturation processes resulting in the generation of a diverse T cell repertoire essential for the establishment of protective immunity.

These progenitors, or thymocytes, are guided through a number of differentiation processes into functional α/β or γ/δ T lymphocytes by thymic stromal cells and the cytokines they produce. The spatial context of thymocyte development is vital (Figure 1.2). Lymphoid progenitors enter the thymus through large venules deep inside the organ near the cortico-

medullary junction; at this stage they are $CD25^-$ $CD44^+$ and are double negative for CD4 and CD8 (DN1). After the DN1 stage, they migrate outwards into the mid cortex, and become $CD25^+$ $CD44^+$ (DN2). The progenitors undergo T cell receptor (TCR) beta chain rearrangement, migrate into the outer cortex and lose expression of CD44 (DN3). Thymocytes in the cortex are highly motile (55, 56), pausing to interact through their TCR with peptide-MHC complexes that are expressed by stromal cells, cTECs, and dendritic cells (55). Notch-mediated signals delivered by binding of Delta ligands (57, 58) on cortical epithelial cells, and chemokines, CXCR4, CCR7 and 9, are required for the outward movement to the subcapsular zone (59-61). To progress from the DN3 to DN4 stage, thymocytes must have a functional pre-TCR expressed on the surface to survive (62).

The TCR is responsible for recognising antigens bound to major MHC molecules. It consists of variable and constant domains which are encoded by different gene segments. The TCR α chain is generated by VJ recombination whereas the β chain is generated by V(D)J recombination, whereby gene segments are randomly joined to generate the complete TCR chain. There are multiple genes for the variable regions and by random joining of these gene segments it is possible to generate an enormous TCR repertoire. However, not all will be functional, self-restricted and self-tolerant and will be eliminated (63, 64).

Through positive selection, immature $CD4^+CD8^+$ DP thymocytes expressing an α/βTCR are rescued from programmed cell death. If successful, they will downregulate CD4 or CD8 expression and become a single positive (SP) thymocyte and migrate to the medulla (56). Success depends on the strength and length of signal with which the TCR binds peptide-MHC expressed on cTECs. In the medulla, T cells that recognise self peptide-self MHC molecules presented by mTECs or DCs with high affinity and avidity will also undergo apoptosis, preventing the maturation of auto-reactive T cells (65, 66). A final mechanism, termed neglect, ensures that if they have a functional TCR that can interact with self-MHC peptide complexes at low affinity, the thymocyte fails to receive a survival signal and dies. More than 95% of developing T cells will die through neglect or negative selection.

T cells spend approximately twelve days in the medulla before egress into the periphery (67). During this period, they undergo a maturation process, identified by expression of CD62L and loss of CD69, to acquire the functional capacity of mature but naïve T cells (68-70). Once exported from the thymus into the circulation, T cells populate the peripheral lymphoid organs. Emigration of T cells from the thymus is regulated by sphingosine-1-phosphate (S1P) and S1P receptor 1 (S1P1). S1P is produced by hematopoietic cells and non-hematopoietic cells including the vascular endothelium (71) and is present at a higher concentration in blood than in

the thymus (72). Conversely, the S1P1 receptor is highly expressed on mature lymphocytes, and is upregulated during the last stage of thymocyte development (73-75). Expression of integrin α5β1 by mature thymocytes (76), the downregulation of CD69 expressed by semi-mature thymocytes (77), and the repulsion from CXCL12, a chemokine abundantly expressed in the thymus (78, 79), are also required for egress of T cells into the periphery.

1.7 Epithelial Cells

In addition to the haemopoietic cells, the thymic parenchyma is also comprised of blood vessels, connective tissue and epithelial cells, which form a highly specialised microenvironment (80, 81). Thymic stromal cells are composed of thymic epithelial cells (TECs), mesenchymal cells, endothelial cells, and hematopoietic cells such as dendritic cells (DCs) and macrophages (82). Thymic epithelial cells compartmentalise developing thymocytes and support distinct stages of T cell development (79). TECs produce IL7, a cytokine that is essential for survival of DN thymocytes (83). They also produce IL-1, IL-6, Stem Cell Factor (SCF) and Tumour Necrosis Factor alpha (TNFα) that promote thymocyte development (for review see (84).

The two subpopulations of TECs, cortical TEC (cTEC) and medullary TEC (mTEC) are so named because of the areas of the thymus in which they are

restricted to. They are differentiated based on their expression of intermediate filament proteins, Keratin (K)8 and K5. Whereas the cortex, $K8^{+}K5^{-}$, is a site that promotes immature T cell development, the thymic medulla, $K8^{-}K5^{+}$, contains mature T cells and supports their interactions with DCs. Both subsets of TECs derive from common progenitors cells that are $K8^{+}K5^{+}$, and are generated from the endoderm of the third pharyngeal pouch (85-88), a process that is dependent upon transcription factors including Tbx1, Hoxa3, Pax1 and Foxn1 (18, 22, 89, 90).

Cortical TECs appear to require interactions with thymocytes committed to the T cell lineage, whilst medullary TECs require $\alpha\beta TCR^{+}$ thymocytes (91). The bifurcation that gives rise to cTECs and mTECs occurs in the absence of lymphocytes, thus, until E11.5 all TECs are homogenous (92). The signal for bifurcation is unknown. The medullary lineage differentiates further under the control of lymphotoxin and TNF signals and arrives at a postmitotic stage characterised by AIRE expression. Both subsets play a crucial role in the development and repertoire formation of T cells (93), and, in turn, are supported by developing thymocytes, via crosstalk (94-97).

In mice that carry a transgene encoding human CD3ε, and thus are deficient for thymocyte development beyond the DN1 stage, TECs are arrested at an immature stage where they are $K5^{+}$ and $K8^{+}$ and are unable to differentiate into $K5^{-}K8^{+}$ cTECs (98). The cortex of thymi from these mice is histologically

abnormal and contains large cysts. In contrast, $K5^{-}K8^{+}$ cTECs are generated and the cortex appears histologically normal in mice that are deficient for thymocyte development beyond the DN3 stage such as recombination-activating gene 1 (RAG1)-deficient mice (98). Therefore signals emanating from DN1-3 thymocytes are required for differentiation of cTECs to form the cortex (94, 99-101).

On the other hand, formation of the medullary areas is dependent on thymocytes which have undergone positive selection (96, 102, 103). This process is unclear, but molecules including Nuclear Factor Kappa-light-chain enhancer of activated B cells (NF-κB), through Lymphotoxin beta Receptor (LTβR), Tumour necrosis factor Receptor-Associated Factor 6 (TRAF6), NF-κB-Inducing Kinase (NIK) and Reticuloendotheliosis viral oncogene homolog B (RelB) are known to be important for this process (104-107). Thus in mice with blocks in later thymocyte development, such as Rag1-deficient mice, the medulla is hypoplastic (41).

Growth and differentiation of all TECs subsets is dependent on the cell-autonomous function of FoxN1, and loss of this transcription factor causes athymia. In FoxN1 deficient mice, TECs cannot progress beyond an early progenitor stage and cannot attract lymphoid progenitor cells, similar to mice which have blocks in thymocyte development. In addition, TECs also require

FGF signals from NC derived mesenchymal cells in order to proliferate and mature (108).

1.8 NC derived mesenchyme

Mesenchyme consists of primitive cells which develop into supportive cells and connective tissues of organs, and is derived from mesoderm or from NCCs. The earliest NCCs are derived from the ectoderm in the region of the neural folds (Figure 1.3A). During neurulation, a region of ectoderm thickens along the axis of the early embryo to form the neural plate, the lateral borders of which curve upward to form the neural folds (Figure 1.3B). As this curling process continues, the folds from each side unite in the midline. The thickened neural epithelium fuses to complete the neural tube, and the ectoderm from each side fuses to form a continuous dorsal covering (Figure 1.3C). NCCs delaminate and migrate ventrally and laterally from the area of the neural folds and differentiate to form a variety of structures (Figure 1.3D). The cephalic NC, the portion from the fifth somite onwards, also contributes a large proportion of cells that differentiate into mesenchymal cells (109).

In order for NCCs to migrate, they must undergo a process of Epithelial to Mesenchymal Transition (EMT). During EMT, NCCs lose their apical-basal polarity characteristic of epithelial cells and acquire a front-rear polarity, required for mesenchymal cell migration. NCCs lose cell-cell adhesions and

undergo cytoskeletal rearrangements and morphological changes that allow them to delaminate and emigrate from the neuroepithelium along three pathways; dorsal, ventral and lateral. They concomitantly acquire migratory ability, with acquisition of cell-surface receptors, metalloproteases and adhesion molecules that allow them to respond to cell-cell interactions and environmental cues that influence their pathway of migration. The process of delamination involves the upregulation of N-cadherin and vimentin, the nuclear localisation of β-catenin and increased production of the transcription factors such as Snail1, Snail2, Twist, EF1/ZEB1, SIP1/ZEB2 and/or E47 that inhibit E-cadherin production (For review see (110)). Once they have reached their destination NCCs differentiate and contribute to pigment cells, most of the peripheral and autonomous nervous systems, the facial skeleton, teeth and aortic arches and participate in the formation of the pharyngeal glands such as the thymus and parathyroids (111-113).

Induction of neurulation is dependent on a number of factors including Wnts, BMPs and FGFs (114-116). Notably, inhibition of BMP signalling in the premigratory rhombencephalic NC blocks either the production or migration of NCCs (116). NCCs are essential for various aspects of vertebrate development (111), and aberrant NCC migration, survival or differentiation contributes to DiGeorge's syndrome (117, 118). Di George's syndrome is characterised by cardiac and facial abnormalities, thymic aplasia, cleft palate and hypocalcaemia. Moreover, specific inactivation of TGFβ signalling in NC

stem cells results in cardiovascular defects and thymic, parathyroid and craniofacial abnormalities, similar to DiGeorge's syndrome. In DiGeorge's syndrome it is though that mutant NCCs migrate into the pharyngeal apparatus but are unable to acquire non-neural cell fates (119).

1.9 NCCs and the Thymus

NCCs have been shown to play a major role in the formation of the thymic rudiment. NCCs were first shown to contribute to connective tissues in the thymic capsule and to surround blood vessels inside the thymus of chick-quail chimeras (13). Cells from these two species can be distinguished due to morphological differences in interphase chromatin organisation. Thus, quail NC was implanted into chick hosts, which showed that the capsule surrounding the developing host thymus was from donor quail NC. More recent experiments using lineage tracing tools have confirmed these results. In the recent experiments, Cre recombinase expressed under the Wnt1 NC specific promoter was used to activate expression of a LacZ reporter inserted into the ubiquitously expressed Rosa26 locus (120). Jiang and colleagues presented evidence that NC derived cells surround the thymus at E13.5 but their numbers decline thereafter and are eventually lost by E17.5 (121).

The functional importance of NC derived cells during thymus organogenesis has been suggested by experiments whereby their removal or reduction in the pharyngeal region results in greatly reduced size or complete absence of the organ (122). Of particular interest are experiments where mesenchyme surrounding the thymic rudiment is removed following trypsin treatment. In these experiments, cultured lobes devoid of such mesenchyme failed to grow, while intact lobes grew and became lobulated. These results indicated that the normal expansion of the epithelial compartment does not occur if NC derived cells are absent (123). Additionally, lobes that were stripped of mesenchyme and cultured overnight in medium containing Bromodeoxyuridine (BrdU) showed a three-fold decrease in BrdU incorporation compared to intact lobes, demonstrating the marked decrease in proliferation of cells in these rudiments (124). Furthermore, ablation of the premigratory cardiac NC in chicks results in disrupted development of the pharyngeal arches, including thymus and parathyroid hypoplasia or agenesis (125).

In addition to the role of FGFs in early thymus development as discussed earlier, FGFs are also required for the proliferation and maturation of TECs, which occurs later in thymus development. Recently, it was demonstrated that NC derived mesenchyme stimulates proliferation and possibly also maturation of epithelial cells, which upon differentiation express molecules required for T cell development (126). Revest *et al* have shown that FGFs,

which are expressed by mesenchymal cells, are important signalling molecules in thymic development (108). Thus, absence of FGFs results in impaired thymic growth due to a lack of epithelial cell proliferation. Additionally, MHCII expression by TECs has also been demonstrated to require mesenchymal cells (127).

In vitro foetal thymic organ culture experiments have shown that NC derived mesenchyme might also be required for the differentiation of lymphoid progenitors into functional T cells (128). These authors, and others, have shown that whilst epithelial cells can support later stages of T cell development, mesenchymal cells are required for their development beyond the $CD4^-$ $CD8^-$ double negative stage (128, 129). Moreover, reaggregate thymic organ culture experiments using E14 lobes showed that both epithelium and non-epithelium stromal cells were required for T cell development from the DN2-DN3 stage (93, 129).

Clearly, early stages of thymus development is dependent upon interactions between epithelial and NC derived cells. However, it has been reported, based on tissue specific heritable genetic labelling, that once epithelial cells have fully gained the competence to support lymphocyte differentiation, and the vasculature of the thymus is complete, the contribution of NCCs in the thymus is greatly reduced or absent (120, 121). Thus, although it has previously been shown that NC derived cells are critically important for the

normal development and function of the thymus, there is controversy regarding their role beyond the early stages of organogenesis.

1.10 Eph/ephrins

Eph receptors and their ligands, ephrins, are members of a family of receptor tyrosine kinases, and are named after the erythropoietin-producing hepatocellular (Eph) carcinoma cell line from which their cDNA was obtained. Eph/ephrin interactions were first recognised as cell guidance cues in vertebrate and invertebrate development. However, it is now known that they are required for a number of developmental processes, such as cell patterning, guidance, migration and adhesion. The mechanisms by which Eph/ephrins control these processes are thought to involve regulation of the actin cytoskeleton by activating members of the Rho family GTPases (reviewed in (130) & (131)). They are expressed in virtually all tissues in the embryo, including the thymus where they appear to have immunoregulatory properties (132-140).

1.11 Structure of Eph/ephrins

Eph receptors comprise a subfamily of receptor tyrosine kinases (RTKs) that interact with cell surface-bound ephrin ligands, which are part of a family of related proteins. There are two classes of ephrins; ephrin-A's, which are tethered to the membrane by a glycosyl phosphatidyl inositol (GPI) moiety, and ephrin-B's, which span the membrane and have a short cytoplasmic tail. The extracellular part of the Eph receptors includes an N-terminal ephrin-

binding domain, a cystein-rich region and two fibronectin type-III repeats. The extracellular domain is separated by a membrane-spanning segment from the cytoplasmic part, which includes a juxtamembrane segment, a tyrosine kinase domain and a sterile a-motif (Figure 1.4A). However, unlike the other Eph receptors, the tyrosine kinase domain of the EphB6 receptor is non-functional. The first step in the formation of a “signalling cluster” is the monovalent interaction between an Eph receptor and an ephrin ligand. It is important to note that interaction between receptor and ligand can occur both *in trans* (between two opposing cells), and may also occur *in cis* (within the same cell) (141).

Both receptor and ligand are capable of transducing a signalling cascade upon interaction, referred to as forward and reverse signalling respectively. Upon binding to an ephrin ligand, Eph receptors activate downstream signalling cascades, termed forward signalling. Additionally, the ligand also has biological activity, known as reverse signalling. Reverse signalling is activated by the transmembrane domain of ephrins, whereby tyrosine phosphorylation of its cytoplasmic tail permits interactions with various signalling molecules. Most Eph receptors and the B-type ephrins also have a carboxy-terminal PDZ domain-binding site, whilst the GPI linked A-type ligands are thought to use associated transmembrane proteins in order to signal (Figure 1.4A). Within the A or B class, interactions between receptor and ligand are promiscuous. Thus multiple receptors can bind to a ligand

and multiple ligands can bind to a receptor, giving rise to redundancy (Figure 1.4B).

1.12 Downstream signalling pathways

Eph/ephrins do not act in isolation; they are part of a complex network of regulatory pathways that act together to control various biological responses. This network of pathways includes cell surface receptors (FGF Receptors, Receptor-like Tyrosine Kinase (Ryk), chemokine receptors); adhesion molecules (integrins, immunoglobulin superfamily (IgSF) proteins, cadherins, claudins); channels and pores (Connexins/innexins, N-methyl-D-aspartic acid (NMDA) receptors); and cell surface proteases (A Disintegrin And Metalloprotease (ADAM), Presenilins) (for review, see (142)).

Of particular interest is a common pathway shared by Eph and ephrins; the Ras/Rho family of GTPases, which regulates the organisation of the actin cytoskeleton and cell adhesion. In this kinase-dependent pathway guanine exchange factors (GEFs) such as Tiam 1, kalirin-7 and intersectin that catalyse the Rho family GTPases Rac1 and Cdc42 into the active state, are phosphorylated by the Eph receptor (143-145). Rho family GTPases regulate the formation of contractile actin-myosin filaments in response to a variety of extracellular stimuli. In particular, Rac induces actin polymerisation at the cell periphery to produce lamellipodia, and Cdc42 promotes actin

filament assembly and filopodia formation. Molecules downstream of Cdc42 include members of the WASP/WAVE family of Arp2/3 complex activators, which promote actin polymerisation (143, 144) (Figure 1.5).

For a cell to migrate it must generate front-rear polarity, a process that is largely controlled by localised activation of the Rho family of small GTPases (146). Cdc42 is a master regulator of polarity (147), and is active towards the front of migrating cells (148). Cdc42 mediates polarity via two mechanisms; restricting where lamellipodia form (149); and localising the microtubule organising centre (MTOC) and golgi apparatus in front of the nucleus, orientated towards to the leading edge (150-152). The latter mechanism involves the Cdc42 effector, Par6, which exists in a complex with Par3 and atypical Protein Kinase C (aPKC) (153). Also downstream of Cdc42 is Pak1, which itself can mediate Cdc42 activation via a positive feedback mechanism (154). Once polarity is established and the cell has formed an active protrusion, the protrusion is stabilised by attachment to the surrounding extracellular matrix via integrins. Integrins preferentially locate to the leading edge of migrating cells (155), and large clusters of integrins are termed focal adhesions. Focal adhesions are also dependent on Rac and Cdc42, which mediate their attachment to the ECM.

1.13 Adhesion/Repulsion

An interesting paradox of Eph/ephrin signalling is that it can be both repulsive and attractive. Forward signalling by activation of Eph receptor tyrosine kinases can lead to repulsion of Eph receptor expressing NCCs. Likewise, Eph expressing axonal growth cones can be triggered to retract away from tissues expressing the corresponding ligand (156-158). However, the same receptor-ligand pairs can also promote adhesion in certain instances. Eph receptor signals of different intensities can produce opposite effects, for example, high concentrations of ephrin-A2 triggers a repulsive response, whereas low concentrations promotes growth of cultured nerve cells (159). Molecules involved in adhesion, such as integrins, are through to act downstream of Eph receptors, controlled, in part, by Focal Adhesion Kinase (FAK) and R-Ras (Figure 1.5).

1.14 Axon guidance

Axons grow towards their target tissues where they form synapses with appropriate cells in order to establish neuronal connectivity. Axons are guided by molecular cues within the extracellular matrix or by signals expressed on the surface of nearby cells. Cells expressing Eph receptors were first observed to avoid territories expressing ephrins, thus providing necessary cues to guide axons to their appropriate targets (160). However, Eph/ephrins can also regulate axon pathfinding through attractive

interactions (161-165), depending on which receptors and downstream signalling pathways are activated.

The trajectories of axons have been shown to be dependent on Eph receptors and ephrins distributed in gradients or forming boundaries (157, 158, 166). Moreover, EphB and B type ephrins are known to regulate multiple steps in assembly and maturation of the pre- and postsynaptic sides of excitatory synapses (167). For example, activation of ephrin-B reverse signalling by postsynaptic EphB2 has been implicated in the morphological and functional maturation of developing retinotectal synapses in Xenopus optic tectum (168). Additionally, the EphB2 extracellular portion also associates with NMDA neurotransmitter receptors and promotes clustering at synapses following ephrin-B stimulation (169).

1.15 Segmentation

In addition to axon guidance, Eph and ephrins are expressed in a segmented pattern in the hindbrain and in the somites, and are implicated in segmentation during embryogenesis (170). In Xenopus, Eph and ephrins are normally expressed in alternating rhombomeres. Thus, expression of a dominant negative form of EphA4 lacking the kinase domain in the hindbrain can disrupt segmental expression of certain genes (171). The sorting of cells at rhombomere boundaries appears to involve forward signalling since a

dominant negative form of ephrin-B2 lacking the cytoplasmic domain was still competent to induce sorting (172). In mouse, EphA4 and ephrin-B2 are expressed in similar expression domains, but unlike in Xenopus, there are no defects in hindbrain segmentation in mice deficient for these molecules, probably due to redundancy (173, 174).

1.16 Angiogenesis

Angiogenic remodelling in the mouse is defective in the absence of ephrin-B2 and its preferred receptor, EphB4 (175-177). EphB4 expression is largely restricted to veins, whilst ephrin-B2 is expressed on arteries, and thus may be involved in determining arterial and venous identity. Reverse signalling through the ligand has been demonstrated to be required for blood vessel remodelling, since expression of a deleted form lacking the cytoplasmic domain was unable to rescue the angiogenic defects associated with the loss of ephrin-B2 (174). EphB4 induced forward signalling is also implicated in regulating angiogenic growth in Xenopus (178). Here EphB4, ephrin-B1 and ephrin-B2 are expressed in complementary patterns in the developing vasculature and somites respectively. Moreover, disruption of the Eph/ephrin interaction leads to defects in intersomitic vessel migration (176, 178). Additionally, overexpression of EphB4 lacking the tyrosine kinase domain induced disorganisation of the intersomitic vascular network, thus forward

signalling is though to be an important player in intersomitic vessel development.

Mural cell association with blood vessels is also dependent on Eph/ephrin interactions. When the *ephrin-B2* gene is inactivated specifically in mural cells, the vessels become leaky leading to oedema and extensive haemorrhaging into the skin (179). In these mice, ephrin-B2 deficient perivascular cells were only loosely attached to vessel walls, made insufficient contact with endothelial cells and failed to envelope the endothelial monolayer. Likewise, mutant vascular Smooth Muscle Cells (vSMCs) showed attachment defects and covered microvessels in a discontinuous manner. Interestingly, these cells also showed defects in spreading, focal adhesion formation and increased migration due to unpolarised motility.

1.17 NC migration

Eph/ephrins regulate migration of cranial and trunk NCCs by repulsion (180, 181). In Xenopus, Eph and ephrins are expressed on adjacent streams of migrating branchial NCCs, thus, repelling those expressing different Eph or ephrin molecules. Moreover, disruption of Eph receptor function results in NCC migration into areas where they would not generally be found. For instance, dominant negative EphA4 mutants induced scattering of NCCs and

allowed them to invade improper territories that they would normally be repelled from. This was demonstrated to be due to cell autonomous signalling since only EphA4 expressing, and not other NCCs, were affected (182). In the mouse, forward signalling also regulates NCC migration into the pharyngeal arches (174).

In ephrin-B2 null mice, there is a defect in NCC migration into the second pharyngeal arch. Thus, the arches were hypoplastic and abnormally vascularised. This was due to a lack of NCC migration into the arch, and was partially rescued by a mutant form of the ephrin-B2 ligand lacking the cytoplasmic domains, thus indicating that forward signalling was responsible for NCC migration into the arch. It has been proposed that ephrin-B2 is expressed in the neural tube, specifically in rhombomeres (r)4 and r6, repulsing Eph-expressing NCCs away into r3 and r5 (183).

Likewise, ephrin-B1 is also required for proper migration of NCCs and, thus, mice deficient for ephrin-B1 have a cleft palate that is attributed to a defect in NCC migration to the second pharyngeal pouch (184). ephrin-B1 deficient NCCs, and those from mice expressing a mutant form of the ligand in which the PDZ-containing domain was disrupted, also invaded improper territories (184). ephrin-B1 null mice display malformations of the axial and appendicular skeleton that includes the asymmetric attachment of ribs to the sternum, lack of joints and polydactyl (185). ephrin-B1 null, or mice with an

NCC specific deletion of ephrin-B1, do not exhibit defects in other pharyngeal pouch-derived organs, such as those from the third pouch, indicating the specificity of ephrin-B1 for migration of NCCs into the second pouch.

Most molecules, including Eph/ephrins, involved in NC migration act by repulsion, allowing NCCs to migrate into permissive areas and repelling them from forbidden areas. Moreover, there is a lack of evidence for the existence of a chemoattractant involved in NC migration. Moreover, cultured NCCs have been shown to migrate with a high directionality even in the absence of external chemoattractants. Several permissive molecules have been suggested, such as fibronectin, laminin or collagen, but there is no evidence that these molecules can control the directionality of NC migration. Instead, a process of chemokinesis has been suggested, whereby a factor stimulates the migration of cells but with no directionality. In contrast, a molecule that is chemoattractant would stimulate both mobility and directional movement along a gradient (186).

Since NCCs migrate with very persistent directionality, either as a mass of migrating cells or individual cells, it is possible that directionality in these cells is controlled by regulation of cell polarity and the cytoskeletal machinery, which controls the formation of cell protrusions. The Planar Cell Polarity (PCP), or non-canonical Wnt, pathway is a likely candidate to control

directional migration in NCCs, since inhibition of this pathway abolishes the directionality of NCC migration.

Usually, the PCP pathway controls the polarity of epithelial tissues. However, it was recently demonstrated that migration of NC derived mesenchymal cells is also regulated by the PCP pathway. Inhibition of several of the PCP factors, including Wnt11, Frizzled (Fz)7, and dishevelled (Dsh), leads to a complete block in NC migration (187). Analysis of NCC migration indicates that they keep in close contact with others whilst migrating (188), thus, it was speculated that cell-cell contacts maintain the integrity of the tissue and coordinates the formation of lamellipodia at the leading edge (186). PCP signalling is required in the context of NCC migration to stabilise cell protrusions through the small GTPases, Rho and Rac (187, 189).

1.18 Eph/ephrin expression in the thymus

A significant number of Eph receptors and ephrin ligands are expressed in the thymus, and are known to have immuno-regulatory properties (see Table 1.2 for pattern and timing of Eph/ephrin expression in the thymus). EphB/ephrin-B molecules have been demonstrated to have important functions in T cell development in a number of *in vitro* and *in vivo* experiments. Addition of EphB2-Fc or ephrin-B1-Fc to foetal thymic organ cultures (FTOC) increases the number of DPs and SPs and increases

apoptosis in all thymocyte subsets, whilst their addition to reaggregate thymic organ cultures (RTOC) disrupts the three dimensional architecture of epithelial cells (190). EphB-Fc or ephrin-B-Fc fusion proteins bind to their respective partners, but, since they lack biological activity, they block signalling downstream of their respective ligand or receptor partner. Additionally, deficiency in EphB2 and B3 receptors results in profound alteration in both the differentiation of T cell precursors and the organisation of the TEC network (191). Moreover, EphB6$^{-/-}$ mice have impaired T cell responses such as lymphokine secretion and proliferation, and develop delayed-type skin hypersensitivity and EAE. This was demonstrated to be not due to defective T cell development, however, but due to a lack of EphB6 on mature T cells (139).

Eph/ephrins may exert their functions by modulation of the actin-cytoskeleton, which influences the TCR-peptide-MHC interaction. Indeed, EphB receptors have been shown to concentrate in lipid rafts in peripheral T cells when the TCR is strongly engaged (136, 137, 139). The role of EphB receptors in this context is to direct the polymerisation of the actin cytoskeleton at the point of TEC contact, and to facilitate the formation of a stable immunological synapse. Moreover, another key process in synapse formation, adhesion (192), also appears to be regulated by Eph/ephrin signalling (158),(157). Formation of an immunological synapse brings together numerous scaffold proteins, and whilst Ephs have not been

demonstrated to be required for raft aggregation, they do appear to be part of the TCR signalling complex, or signalosome, through adapter molecules associated with their intracellular tails, once it has formed. ephrin-B2 has also been demonstrated to translocate to lipid rafts, assisting TCR signalling after being triggered (136). Cytoskeletal rearrangements are pivotal for T cell signalling (193), and this appears to be regulated by Eph/ephrin activation of the Ras and Rac pathways.

Ephrin ligands have also been shown to regulate the homeostatic mechanism that keeps the number of T cells stable, by triggering apoptosis in these cells (138). Additionally, ephrin-B2 has been shown to stimulate T cell proliferation by augmenting stimulation by IFNγ, and, thus, can co-stimulate T cells in the presence of suboptimal TCR ligation. In this respect, ephrin-B2 may function to reduce the threshold for stimulation by suboptimal foreign antigens.

ephrins are also required for chemotaxis during T cell development. Both ephrin-As and ephrin-B ligands modify the chemotactic response of T cells mediated by stromal cell-derived factor 1α (SDF1α) and macrophage inflammatory protein 3β (MIB3β) (194, 195). Therefore ephrins may play additional roles in adhesion and transmigration through HEV endothelial cells into secondary lymphoid organs and to sites of inflammation.

EphA4, expressed by TECs, and its preferred ligands, ephrin-As, expressed by thymocytes, also affects T cell development. The EphA/ephrin-A family appear to have a role in the development of the TEC network since in their absence the three dimensional TEC architecture is disordered, which in turn disrupts the development of thymocytes. By modulation of the thymocyte-TEC interaction, Eph/ephrins were demonstrated to provide the necessary signals for T cell development, and in the absence of these signals, T cells died due to a failure in differentiation (196).

A further role in colonisation of the thymic primordium and intrathymic migration by reverse ephrin signalling has also been suggested (197). However, the function of Eph/ephrins in early organogenesis and morphogenesis is yet to be fully described.

Figure 1.1: Model of thymus organogenesis. A E9.5: Positioning. Pax1/9 and FGF8 (green) are required for pharyngeal pouch formation. Hoxa3 (red) is required for third pouch (p3) axial identity. **B E11: Initiation.** Rudiment outgrowth beings at E11. The Hox-Pax-Eya-Six cascade is required in the endoderm (yellow); Hoxa3, Eya1 and Six1 might also be required in NCCs. **C E11.5-12.5: Outgrowth and Patterning of the Rudiment.** Regionalisation of the rudiment into thymus- and parathyroid-specific domains. Patterning begins at E10 with the expression of GCM2 (red) in the third pouch, controlled at least in part by the Hox-Pax-Eya-Six cascade. High-level expression of Fonx1 (blue) begins at E11.25. **D E12.5 Separation from the Pharynx and Migration of the Rudiment.** Pax9, Alk5, FRS2α and possibly FGF and BMP expression is required for separation from the pharynx. Pax3, FRS2α and Alk5 are required for the separation of the parathyroid and thymus domains. Migration might be controlled by *Hox3* genes expressed by NCCs. (Adapted from Figure 5 of (85)).

Figure 1.2: Thymus structure and T cell development. The thymus is broadly divided into two histologically defined regions, the cortex and medulla, each of which contains several different thymic epithelial cell (TEC) subtypes. In adults, T-cell precursors enter the thymus through the cortico-medullary junction, and then begin a highly ordered differentiation programme, which is linked to migration through the thymic stroma. Thus, uncommitted progenitors, $CD4^-CD8^-$ double negative (DN) 1 cells are found near the cortico-medullary junction; cells differentiate to the DN2 stage and undergo a proliferative clonal expansion; T-cell lineage commitment and onset of TCR β-chain rearrangement occurs in DN3 cells; and the transition from DN to $CD4^+CD8^+$ DP status occurs near the subcapsule. DP cells then migrate back through the cortex and, having differentiated into either $CD4^+$ or $CD8^+$ single positive (SP) cells, into the medulla. Positive selection occurs mainly in the cortex, and requires cortical TECs, whereas negative selection occurs mainly in the medulla, and is mediated by medullary TECs and peripheral dendritic cells (DCs). SP cells that have completed the differentiation programme egress from the medulla to the periphery through the blood vessels. (Adapted from Figure 1 of (85)).

Figure 1.3: The process of Neurulation. A Shortly after gastrulation, the neural plate (yellow) is open. **B** The neural plate folds, bringing together the neural folds (blue). **C** The neural folds fuse, forming the neural tube (yellow). **D** Neural crest cells (NCCs; blue) delaminate away from the neural tube. (Adapted from Figure 1 of (300)).

Figure 1.4: Schematic of Eph receptors and ephrin ligands. A ephrin-A ligands are attached to the cell surface through a glycosylphosphatidylinositol (GPI)-anchor. The ephrin-B ligands are transmembrane proteins with a short conserved cytoplasmic region consisting of a PDZ-binding motif and a kinase domain. Both classes of ephrins have an extracellular Eph-receptor-binding domain. EphA and EphB transmembrane receptors consist of an ephrin-binding domain, a cystein rich region, two fibronectin type-III domains. The cytoplasmic part of the receptors contain a juxtamembrane segment, the kinase domain and a sterile α-motif (SAM). It is important to note that signalling may occur both *in trans* and *in cis*. Not to scale. (Adapted from Figure 1 of (157)). **B** There is promiscuous binding of Eph receptors and ephrin ligands. Interaction of high affinity are indicated in bold; weaker affinities with dashed lines. (Adapted from (301)).

Figure 1.5: Downstream signalling pathways of EphB receptors. A simplified schematic of some of the effectors that bind to EphB receptors are indicated. Pathways of the left are predominantly involved in repulsion, whilst those on the right concern adhesion. (Adapted from (301, 302, 144)).

Table 1.1: Summary of mutant mice with known thymic and parathyroid defects. Table of mice with thymic and parathyroid defects, detailing which genes are disrupted and the associated thymus/parathyroid phenotype.

Table 1.2: Eph/ephrin expression in the murine thymus. Table describing the pattern and timing of Class A and B Eph receptor and ephrin ligand expression in the thymus.

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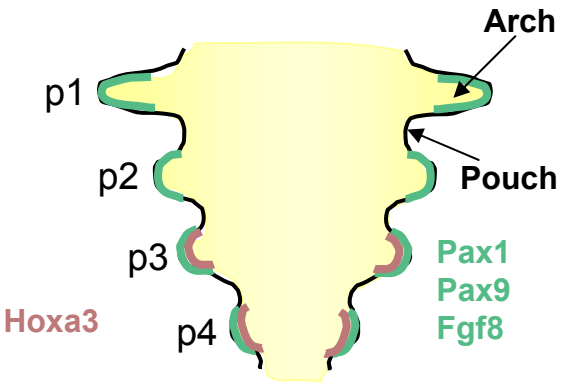
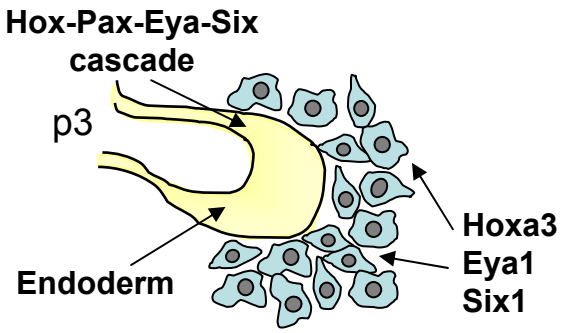
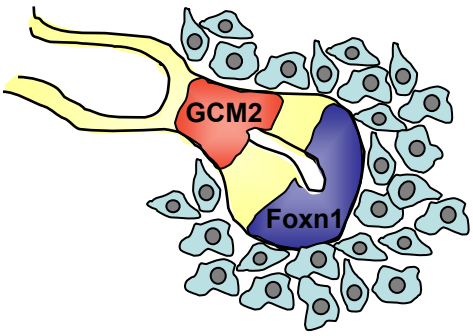


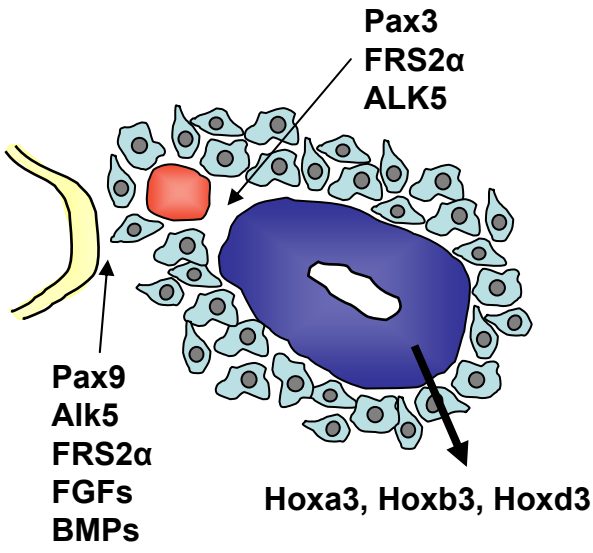
B E11: initiation



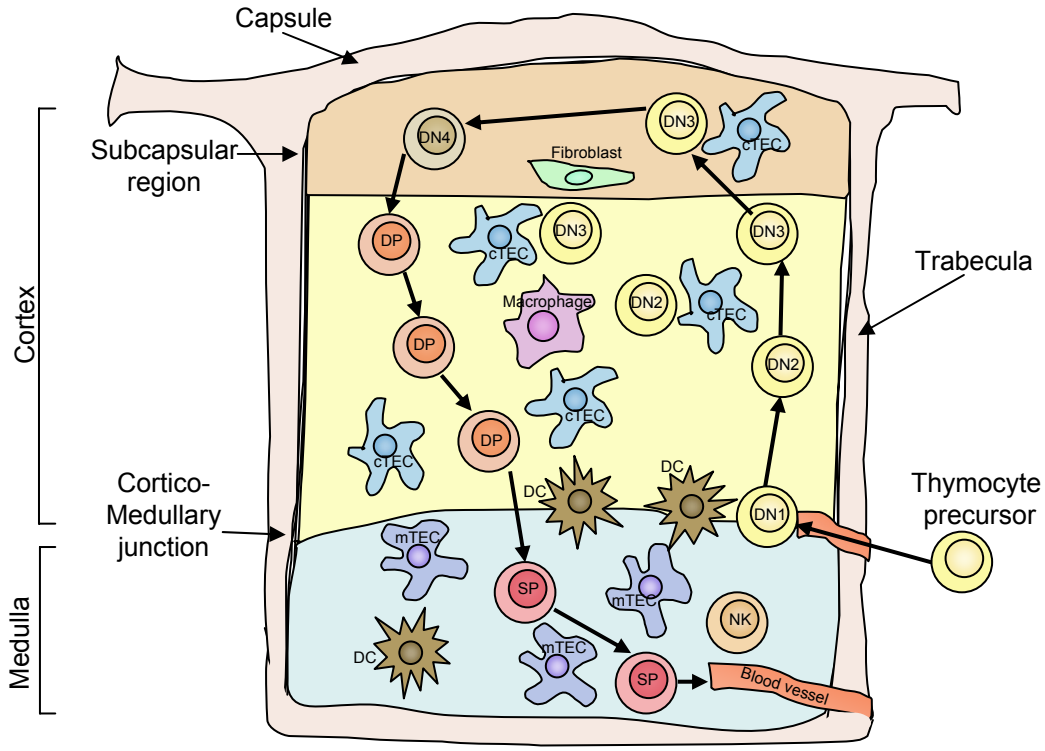
C E11.5-12.5: outgrowth and patterning



D E12.5: separation



NCC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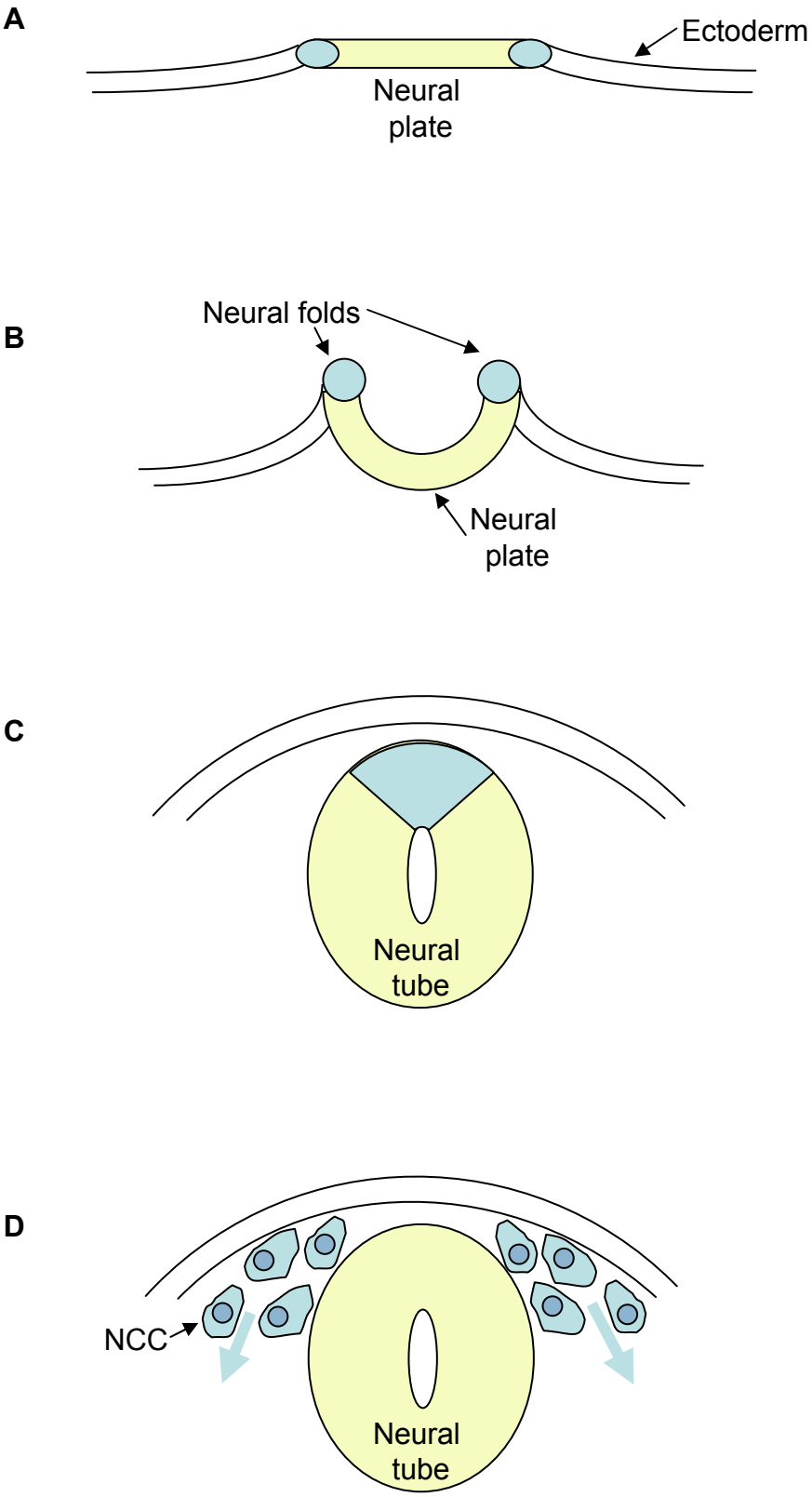
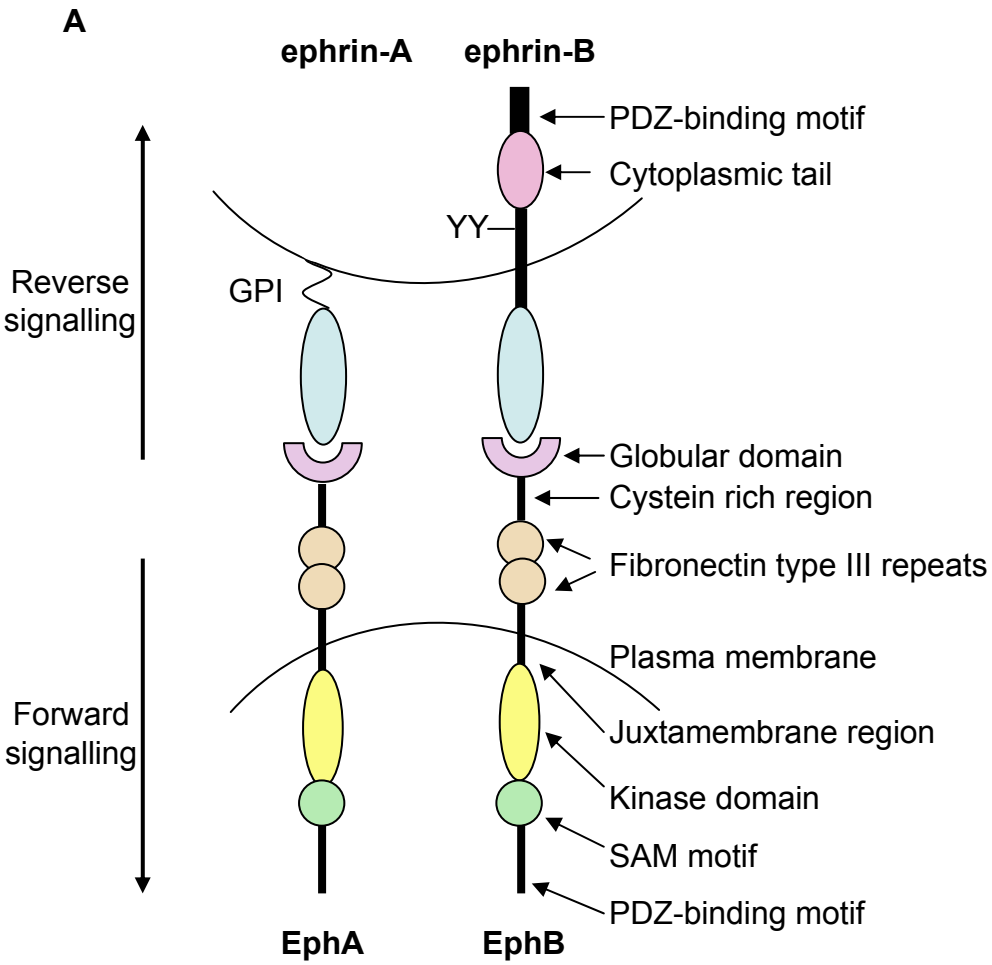
Subcapsular region
Cortex
Medulla
Cortico-Medullary junction
Trabecula
Thymocyte precursor
Fibroblast
Macrophage
Blood vessel
DN4
DN3
DN2
DN1
DP
SP
cTEC
mTEC
DC
NK


A
Ectoderm
Neural
plate
B
Neural folds
Neural
plate
C
Neural
tube
D
NCC
Neural
tube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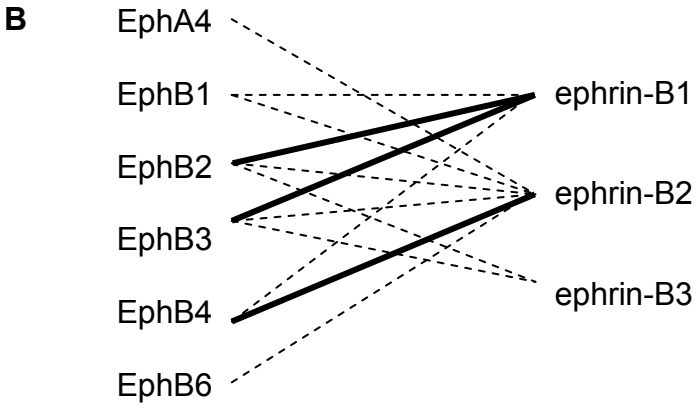
ephrin-A
ephrin-B
PDZ-binding motif
Cytoplasmic tail
YY
GPI
Reverse signalling
Globular domain
Cystein rich region
Fibronectin type III repeats
Plasma membrane
Forward signalling
Juxtamembrane region
Kinase domain
SAM motif
PDZ-binding motif
EphA
EphB


B
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EphB1
EphB2
EphB3
EphB4
EphB6
ephrin-B1
ephrin-B2
ephrin-B3

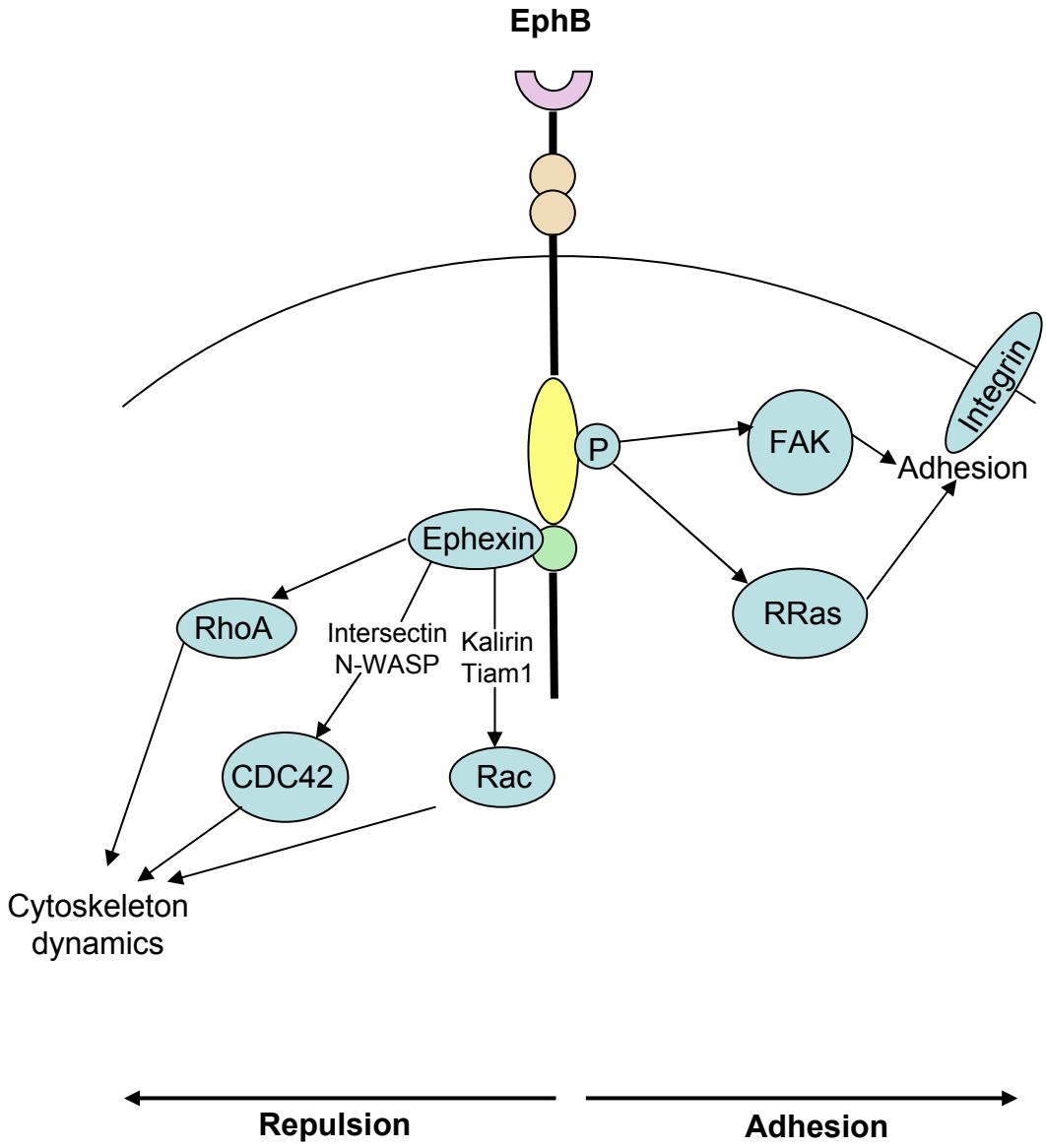

EphB
P
FAK
Integrin
Adhesion
Ephexin
RhoA
Intersectin
N-WASP
Kalirin
Tiam1
RRas
CDC42
Rac
Cytoskeleton
dynamics
Repulsion
Adhesion

Table 1: Summary of mice with known thymic defects

Gene Mutated	Thymus/Parathyroid Phenotype	Ref
Hoxa3	Failure of initial thymus and parathyroid organogenesis	(3, 22)
Pax1	Thymic hypoplasia and mild thymocyte defects; Pax1/Pax9 double mutants have defective pouch formation	(4)
Pax9	Thymus is ectopic and hypoplastic, possible defect in γδ-TCR T cell development	(21)
Splotch (Pax3 null)	Thymus is ectopic due to delayed separation, abnormal boundary between thymus and parathyroid	(23)
$Hoxa3^{+/-}$ $Hoxb3^{-/-}$ $Hoxd3^{-/-}$ compound mutant	Hypoplastic thymi, delayed separation from the pharynx leading to ectopic thymi	(22)
$Hoxa3^{+/-}$ $Pax1^{-/-}$	Ectopic, hypoplastic thymi, due to delayed separation of the thymus/parathyroid primordia from the pharynx	(5)
Pbx1	Absent or hypoplastic primordia, delayed expression of organ-specific markers, reduced proliferation of TECs	(249)
Eya1	Failure of initial thymus and parathyroid organogenesis	(7)
Six1	Patterning of the third pouch into thymus	(8)

	and parathyroid domains occurs normally, but there is a failure to maintain Gcm2 and Foxn1 expression, thus complete disappearance of the thymus/parathyroid by E12.5	
Foxn1	Initial development of the thymus is normal, but arrests at E11.5-E12.5, no colonisation of primordium by lymphocytes	(89, 293-295)
Tbx1	Thymic aplasia or severe hypoplasia	(296)
Endothelin-1	Hypoplastic and ectopic thymus	(297)
Alk5 (TGFβ type-1 receptor)	Ectopic thymi and delayed separation of the thymus and parathyroid rudiments	(25)
$FRS2^{SH2/SH2}$	Ectopic thymus, hypoplastic parathyroid, no separation from the pharynx and of thymus and parathyroid from each other	(26)
FGF8	Thymic agenesis or ectopia, delayed separation from the pharynx	(298)
BMP	Hypoplastic and ectopic thymus	(299)

Table 2: Eph/ephrin expression in the murine thymus

Eph/ ephrin	Pattern of Expression	Timing	Ref
EphA1	Low levels on stromal cells in medulla. (not on thymocytes)	Adult	(135) (284, 285)
EphA2	Low levels on stromal cells in medulla (not on thymocytes). Dendritic cells	Adult	(135) (286, 287)
EphA3	SP Thymocytes and stromal cells in medulla	Adult	(135) (251, 288)
EphA4	SP thymocytes	Adult	(135)
EphA5	(Not on thymocytes)	Adult	(135, 251)
EphA6	All thymocyte subsets	Adult	(135)
EphA7	Stroma of cortex and subcapsule, all thymocyte subsets	Adult	(135)
EphA8	Stroma of subcapsule, scattered SPs in medulla	Adult	(135)
ephrin-A1	Subcapsula region, less in cortex, not in the medulla. All thymocyte subsets. Connective tissue septae	Later foetal stages, and adult.	(135, 285)
ephrin-A2	Throughout the thymus, but less in the medulla. All thymocyte subsets.	Adult	(135, 251)
ephrin-A3	All thymocyte subsets.	Adult	(135, 251)
ephrin-A4	All thymocyte subsets.	Adult	(135)
ephrin-A5	Most highly expressed in subcapsule, less in vicinity of the blood vessels, not in the medulla. All thymocyte subsets.	Adult	(135) (251)
EphB1	Dendritic cells. Broadly detected in thymus	Adult	(288, 289)
EphB2	Total thymocytes, thymic epithelium (cortex and medulla) stroma and thymocytes	Foetal and adult	(132, 191, 195)
EphB3	Total thymocytes, thymic epithelium (cortex and medulla) stroma and thymocytes	Foetal and adult	(191, 195)
EphB4	Broadly detected in thymus	Adult	(290)
EphB6	Mature T cells, also DPs	Upregulated in $CD4^+$ $CD8^+$ SPs after	(133, 134, 252) (291)

		stimulation. Adult	
ephrin-B1	Total thymocytes, thymic epithelium (cortex and medulla) stroma and thymocytes	Foetal and adult	(138, 140, 191, 291, 292)
ephrin-B2	Total thymocytes, thymic epithelium (cortex and medulla) stroma and thymocytes. Less in cortico-medullary junction. Monocytes and macrophages	Foetal and adult	(136, 138, 140, 191, 291, 292)
ephrin-B3	All thymocyte subsets, especially $CD8^+$ SP	Adult	(136, 138, 140)

Chapter Two: Materials and Methods

2.1 Chemicals and Reagents

Chemicals were obtained from Sigma-Aldrich and VWR International Ltd. Antibodies were purchased from Invitrogen, eBiosciences, R&D systems, Abcam, BD Pharmingen, DAKO, BD Biosciences and Sigma-Aldrich (see Table 2.1). Microscope slides, coverslips, and other microscopy supplies were obtained from Agar Scientific and MatTek. Tissue culture plastics were obtained from Corning-Costar, Nalge Nunc International and Beckton Dickinson. Media, Foetal Calf Serum and other supplements were obtained from GlobePharm, Gibco and Sigma-Aldrich. Proteinase K, PCR and gel electrophoresis reagents were purchased from Thermoscientific, Amersham Biosciences, Biowhitaker Molecular Applications and Sigma-Aldrich. Custom-made oligonucleotides were obtained from Eurofins MWG Operon and Sigma Genosys.

2.2 Mice

Wnt1-Cre (198), *Sox10-Cre* (199), *IL7-Cre* (200), *Rosa26*^{eYfp} (201), *EphrinB2*$^{Lx/Lx}$ (202), *DIP-R* (Williams A, PhD Thesis 2007), and C57BL/6 mice were bred at the animal facilities in MRC-NIMR. *EphrinB2*$^{\Delta V/\Delta V}$, *EphrinB2*$^{5Y/5Y}$ (203) and *PDGFRβ*$^{-/-}$ (204) mice were bred at the animal facilities in the London Research Institute, Cancer Research UK, London. All mice were bred and kept under specific pathogen free conditions.

Experiments were carried out according to the UK Home Office rules for animal experimentation.

2.3 Genomic DNA preparation

5mm of tail was clipped and put into a sterile eppendorf tube containing 500µl of tail lysis buffer and 10µl proteinase K (10mg/ml), and incubated at 55ºC overnight. After centrifugation at 13000rpm in a benchtop centrifuge for 30 minutes, the supernatant was transferred to a fresh tube and mixed with 1ml isopropanol at -20ºC for 10 minutes to extract the DNA. After a second centrifugation step, the supernatant was removed and 70% ethanol added to wash the remaining pellet. The pellet was resuspended in double distilled H_2O to a concentration of 100ng/µl, as determined by spectrophotometry according to the following formula;

[DNA]ug/ul = (Abs 260x50xdilution) / 1000

Tail Lysis Buffer;

100mM Tris-HCL pH 8.5; 5mM EDTA; 0.2% SDS; 200mM NaCl

2.4 Genotyping

100ng of DNA was added to a mix of 1x Reaction Buffer IV, 10nm dNTP's, 25mM magnesium chloride solution, 50pmol/µl of each appropriate primer in

a final volume of 20µl (See Table 2.1 for PCR primers). The PCR was carried out in a DYAD PCR machine and the products analysed by agarose gel electrophoresis.

2.5 Agarose Gel Electrophoresis

1% agarose gels were prepared by heating agarose powder in 1xTAE until boiling, and then adding 0.5µg/ml ethidium bromide. Orange G Loading Dye was added at 1:5 volume of PCR products and loaded into an agarose gel and run horizontally submerged in a tank containing 1x TAE at 85V. DNA bands were observed and photographed under short wave UV light.

50x TAE

2M Tris-Acetate, 100mM EDTA pH 8.0

Orange G Loading Dye

20% ficoll, 110mM Tris-HCl, pH 7.5, 0.25% (w/v) Orange G Dye

2.6 BrdU administration

Pregnant female mice were given a pulse of one intraperitoneal injection of BrdU (66µg/g of mouse) or PBS at E13.5, E14.5 or E15.5. Embryos were recovered two days later, and microdissected to remove the thymic

rudiment. After fixation in 4% PFA for 30 minutes at RT, thymi were incubated with 2M hydrochloric acid and washed with borate buffer before staining with an antibody recognising BrdU, and analysed by confocal microscopy.

Borate Buffer;

6.18g boric acid; 1L H_2O, pH 8.5.

2.7 Whole-mount Immunohistochemistry

Embryos and organs were fixed in 4% PFA at 4ºC and washed in 0.15% PBS-Triton-X100. 100µm sections were cut after embedding the fixed organs in 8% agarose and using a Vibratome (Leica Microsystems). Sections were washed and blocked in 10% serum (species of serum appropriate to the secondary antibody used) and subsequently stained with antibodies (See Table 2.2). Embryos negative for *Cre* resulting from *Wnt1-Cre;Rosa26*^{eYfp} x C57Bl/6 breedings were stained with an antibody detecting eYFP and used as negative controls. Background autofluorescence was collected by excitation with 488nm laser and emission collected between 490nm and 515nm.

Paraformaldehyde;

4g PFA powder; 90ml H_2O; 10ml 10x PBS. pH 7.3 with HCl.

8% Agarose;

4% low melting point agarose; 4% high melting point agarose; H_2O.

2.8 Frozen section preparation and staining

Tissues were embedded in Optical Cutting Temperature (OCT) compound (Tissue-Tek, VWR), snap frozen on dry ice and stored at -80ºC. Sections (10µm) were cut using a cryostat (Leica CM1900) and collected onto poly-L-lysine coated glass slides. Sections were air dried for 20 minutes before being fixed for two minutes in 100% acetone (-20ºC) and air dried for a further 20 minutes. Sections were blocked in 5% serum and subsequently stained with antibodies. Slides were rinsed with water and air dried before mounting with Vectashield Hardset mounting compound (VWR).

2.9 Microscopy

Embryonic and adult tissues and organs were stained in wholemount using antibodies described in Table 2.1. Tissues were fixed in 4% PFA at 4ºC, washed in 0.15% PBS-Triton-X100 prior to blocking non specific antibody staining with serum of species appropriate to the secondary antibody used. Samples were incubated with primary antibody for 4 hours at room temperature, or at 4ºC overnight. After washing, the samples were stained

with a fluorescently labelled secondary antibody, washed and fixed with 4% PFA. Fluorescence was detected by stereo or confocal microscopy

Stereo Microscopy

eYFP expression was analysed using a Zeiss M2Bio (Carl Zeiss) stereo-fluorescent microscope. Pictures were acquired with an Orca ER (Hammamatsu) camera and Open Lab software (Improvision). eYFP expression was analysed using a wide band GFP filter cube (470/500LP) (Krammer scientific) with Green (525) and Red (630) filters (Chroma). Images were merged in OpenLab (Improvision) and contrast enhanced in Photoshop (Adobe).

Confocal Microscopy

For detection of immunofluorescence, samples were analysed using a Leica SP2 confocal, or Leica AOBS confocal microscope (Leica). Tissue stained in wholemount was dehydrated in methanol and optically cleared using Benzyl Alcohol and Benzyl Benzoate (BABB, 1:2 dilution) before imaging. Confocal images are presented as single sections, as a merge of a number of serial sections, or a three-dimensional rendering of many serial sections. Three-dimensional renderings were generated in Volocity software (Improvision).

2.10 Cell Migration assay

Foetal thymic lobes were isolated from E13.5 *Wnt1-Cre;Rosa26*^{eYfp} embryos obtained from timed matings of *Wnt1-Cre;Rosa26*^{eYfp} heterozygous males with C57BL6 females and *Ef.B2*$^{-/-}$*;Wnt1-Cre;Rosa26*^{eYFP} embryos from *EF.B2*$^{Lx/-}$ with *Ef.B2*$^{Lx/Lx}$*;Wnt1-Cre;Rosa26*^{eYFP} breedings. Transgenic embryos were identified by YFP expression as observed by stereo microscopy. The lobes were digested with 1mg/ml Collagenase D in Air Buffered Iscove's Modified Dulbecco's Medium (AB-IMDM) at 37ºC for 30 minutes, washed and resuspended in 250µl IMDM supplemented with 10% heat inactivated Foetal Calf Serum (FCS), 2mM L-Glutamine, 0.06mg/ml penicillin and 0.1mg/ml streptomycin. Glass bottom culture dishes were incubated with medium at 37^{o}C for 15 minutes and then removed by aspiration. 250uL of single-cell suspension prepared from foetal thymi was added to each microwell onto the glass surface. The cells were left to attach to the glass by incubation at 37^{o}C for 1 hour. Subsequently, 2mL of medium was added to the dish and cultured for two hours or overnight. Subsequently, the medium was removed, washed twice and incubated with serum-free medium for two hours. Cell movements were imaged with a DeltaVision widefield fluorescent imaging system for four hours and analysed with softWoRx (Applied Prescision) and Volocity (Improvision) software packages. Results are presented as total displacement and meandering index, a measurement of directional movement where 1 = migration in one direction and 0 = totally random movement.

2.11 Foetal Thymic Organ Culture

Foetal thymic lobes were isolated from E13.5 *Wnt1-Cre;Rosa26*^{eYfp} embryos obtained from timed matings of *Wnt1-Cre;Rosa26*^{eYfp} heterozygous males with C57BL/6 females (198). Transgenic embryos were identified by eYFP expression. Lobes were transferred to Nucleopore polycarbonate membranes and cultured at 37ºC, 5% CO_2 in Roswell Park Memorial Institute (RPMI)-1640 supplemented with 10% heat inactivated FCS, 2mM L-glutamine, 0.06mg/ml penicillin, 0.1mg/ml streptomycin, and 50μM 2-Mercaptoethanol. Inhibitors of blood vessel development, DC101 antibody and Sunitinib Malate, were added at 40μg/ml and 50ng/ml respectively. Biotinylated anti-human CD2 (IgG1) 40μg/ml was used as a control for DC101. Negative control for Sunitinib Malate was no addition of biochemical inhibitor. The lobes were left in culture for four days before fixation in 4% PFA at RT for 15 minutes. Subsequently, the lobes were stained with antibodies and imaged by confocal microscopy.

2.12 Whole Organ Culture

Thymic rudiments from E13.5 control *Wnt1-Cre;Rosa26*^{eYfp} and mutant *Ef.B2*$^{Lx/Lx}$*;Wnt1-Cre;Rosa26*^{eYFP} embryos were dissected and embedded into a collagen gel. The collagen gel was prepared by diluting a concentrated stock of collagen in acetic acid and 10x DMEM to the desired concentration

(1mg/ml). Using an aliquot of the appropriate volume of the diluted collagen solution, the pH was neutralised to pH7.5 with 1M sodium hydroxide. 500µl of neutralised collagen stock was pipetted into a 8-multi-well slide. The thymi were embedded into the collagen and left to set for 2 hours at 4°C. The gels were then incubated for 48 hours at 37°C before fixing with 4% paraformaldehyde for 15 minutes. The paraformaldehyde was quenched with 0.15M glycine for 10 minutes before washing three times in PBT for 10 minutes each. Once washed, the gels were stained with an anti-eYFP antibody as described for whole-mount staining, and imaged by confocal microscopy.

Collagen Gel (5ml);

500µl 10x DMEM (with sodium bicarbonate)

648µl Rat tail Collagen Type I (9.03mg/ml)

3.852ml 0.02M acetic acid

PBT;

0.1% Triton X-100 in phosphate buffered saline

2.13 Flow Cytometry

Single cell suspensions were obtained from the thymus or spleen by digestion with 1mg/ml collagenase D for 1 hour at 37°C, or mashing through

a cell strainer. $1x10^6$ cells were stained in 100ul ice-cold PBS supplemented with 0.5% BSA and 0.02% sodium azide. Cells were incubated with appropriate antibodies for 30 minutes on ice before washing twice with 100ul PBS azide (See Table 2.1 for list of antibodies). Prior to staining with intracellular antibodies, cells were fixed in 2% paraformaldehyde and permeabilised with 90% Methanol. Samples were acquired on a FACS Calibur (BD Biosciences) with Cellquest software and analysed in FlowJo (TreeStar).

2.14 Embryo preparation

C57BL/6 females were mated with *Wnt1-Cre;Rosa26*^{eYFP} males, and *EF.B2*$^{Lx/-}$ females were mated with *Ef.B2*$^{Lx/Lx}$*;Wnt1-Cre;Rosa26*^{eYFP} males, and the embryos dissected and collected at 11.5 – 13.5 days of gestation. The litter was screened by stereomicroscopy to detect YFP^+ transgenic embryos. Tails were taken from the embryos and digested with Proteinase K (PK) to extract DNA for genotyping. The embryos were fixed with 4% PFA at 4ºC overnight, then washed in PBS containing 0.1% Triton-X 100 three times, dehydrated in increasing concentrations of methanol in PBS and stored at -20ºC.

2.15 Probe preparation

DNA sequences from *FoxN1* and *GCM2* genes were ligated into polylinker sites of pBSII SK and T-Easy (Promega) vectors respectively. The vectors were linearised at a suitable site and “run-off” transcripts were produced by PCR incorporating DIG labelled UTP.

2.16 *In situ* Hybridisation

Embryos and tissues were fixed in 4% PFA at room temperature. Fixation times varied depending on the thickness of the tissue sample, or embryonic stage. Embryos were washed in PBT three times over 15 minutes and incubated in 6% hydrogen peroxide for 1 hour at room temperature. Samples were washed three times over 15 minutes and incubated in 10µg/ml proteinase K in PBT at room temperature. The activity of proteinase K was quenched by incubating the samples with 2µl/ml glycine in PBT and fixed in 0.2% gluteraldehyde in 4% PFA for 20 minutes at room temperature. The samples were incubated in hybridisation buffer, which was prewarmed to 70ºC, for one hour at 70ºC. The samples were then transferred to fresh hybridisation buffer with 10mg/ml torula yeast RNA, 10mg/ml herring sperm DNA and DIG labelled denatured probe (1:100-1:200 dilution) and incubated at 70ºC overnight in a humidified chamber.

The samples were washed twice in solution I for 30 minutes at 70ºC, followed by one wash with solution I:II for 10 minutes at 70ºC, three washes

with solution II for 5 minutes each at room temperature before two 30 minute incubation steps with RNAase A (100µg/ml) in solution II at 37ºC. After the second incubation step, the samples were washed once with solution II, then once with solution III for 5 minutes at room temperature, followed by two washes with solution III for 30 minutes at 65ºC. The samples were then washed three times with TBST, each for 5 minutes at room temperature and preblocked with 10% sheep serum in TBST for 1 hour at room temperature. The samples were incubated overnight rocking at 4ºC with anti-DIG-AP FAB fragments (1/2000 dilution) in 1% sheep serum in TBST.

PBT;

Phosphate buffered saline; 0.1% tween 20.

Hybridisation buffer;

6ml Formamide; 3ml 20x SSC; 2.98ml H_2O ; 12ul Tween 20 ; 6ul Heparin (100mg/ml).

Solution I;

10ml Formamide; 4ml 20x SSC pH4.5; 2ml 10% SDS; 4ml H_2O. Prewarmed.

Solution II;

20ml 5M NaCl; 2ml 1M Tris pH7.5; 200µl Tween 20 ; 177.8ml H_2O.

Solution III;

10ml Formamide; 2ml 20x SSC pH4.5, 8ml H_2O. Prewarmed.

TBST;

4g NaCl; 0.1g KCl; 12.5ml 1M Tris pH7.5; 0.1% Tween 20 in 500ml H_2O.

Following overnight incubation, the samples were washed six times with TBST for one hour each at room temperature, and then overnight at 4ºC, rocking.

After washing overnight, the samples were washed with NTMT three times for 10 minutes each at room temperature, then incubated with 4.5µl NBT and 3.5µl BCIP per ml of NTMT in the dark at room temperature. The reaction was stopped with two washes with NTMT, then washed with PBT before fixation with 4% PFA in 0/1% gluteraldehyde and transferred to 0.1% PFA/PBT. Sections were cut and imaged by wide-field light microscopy.

NTMT;

3ml 5M NaCl; 15ml 1M Tris pH9.5, 7.5ml 1M $MgCl_2$; 150µl Tween 20; 124.35ml H_2O.

Table 2.1. Antibodies used in Immunohistochemistry and Flow Cytometry

Table 2.2 PCR Primers for Genotyping

Antibodies

Antibody	Conjugate	Company
BrdU	Alexa Fluor 568	Invitrogen
GFP	Alexa Fluor 488, 594, 647	Invitrogen
Endomucin	Un-conjugated	Prof D. Vestweber
αSMA	Cy3	Abcam
PDGFRα	Biotinylated	R&D Systems
PDGFRβ	Biotinylated	R&D Systems
Anti-rat IgG	Alexa Fluor 488, 594	Invitrogen
Anti-mouse IgG	Alexa Fluor 568, 594	Invitrogen
Anti-rabbit IgG	Alexa Fluor 594	Invitrogen
CD31	Un-conjugated	BD Pharmingen
PanK	Un-conjugated	Dako
CD45	PE	eBiosciences
MHCII	PE	BD Biosciences
CD31	APC	eBiosciences
Ng2	Un-conjugated	Sigma-Aldrich
Anti-mouse IgG	PE	eBiosciences
Streptavidin	APC	eBiosciences
Streptavidin	PE	eBiosciences
Anti-DIG-AP Fab Fragments	Alkaline Phosphatase	

Gene	Fragment size (bp)	Anneal °C	Forward primer	Reverse primer
Cre (Wnt1, FoxN1)	250	62	TTCCCGCAGAACC TGAAGATGTTCG	GCCAGATTACGTATA TCCTGGCAGC
eYFP	200	62	AACATCGAGGACG GCAGCGT	GGTCACGAACTCCA GCAGGA
EphrinB2 Lx	WT 270 Lx 370	62	CTTCAGCAATATAC ACAGGATG	TGCTTGATTGAAACG AAGCCCGA
EphrinB2 KO	KO 370	62	CTTCAGCAATATAC ACAGGATG	AATACTGTTACTACA GGGTCC
EphrinB2 (ΔV/5F) KI	KI 500	62	CGTCCAGAGCTAG AAGCTGGTA	GGTACACTTACCTG GTACCCCAC
EphrinB2 (ΔV/5F) WT		62	CTCTGTGTGGAAG TACTGTTG	CCGCCAATGTGTGT CTGTAGC
Rosa-DTA	200	58	GACGCTGCGGGAT ACTCTGT	CTGAGCACTACACG CGAAGC

Chapter Three: Characterisation of NC derived cells in embryonic and adult thymus

In this chapter, the current hypothesis that NC derived cells only play an important role in the early stages of thymus organogenesis, and are lost or replaced by cells of other mesenchymal origin at later stages, is challenged. Instead, evidence is presented which shows that NC derived cells persist in the thymus beyond birth and remain there up to nine to ten months of age. Moreover, these cells differentiate into pericytes and smooth muscle cells and surround blood vessel endothelium, indicating a further role of NC derived cells in thymic blood vessel development and function.

3.1 Experimental Rationale

Neural crest cells (NCCs) have been shown to play a major role in the formation of the thymic rudiment. NCCs migrate extensively from the neural folds and contribute to various cell populations in different tissues throughout the embryo (112, 113, 122). Interestingly, NCCs were shown to contribute also to connective tissues in the thymic capsule and to surround blood vessels inside the thymus of chick-quail chimeras (13).

Between E9.5 and E12.5 the development of the murine thymus is dependent upon interactions between epithelial and neural crest (NC) derived cells. NC derived mesenchyme stimulates proliferation and possibly also the maturation of epithelial cells, which upon differentiation express among other molecules, SCF, Delta-Like Ligand 4 (DII4) and Immune response (Ia) antigen, required for T cell development (126). Additionally, Revest *et al* have shown that fibroblast growth factors (FGFs), which are expressed by mesenchymal cells, are important signalling molecules in thymic development (108). These authors showed that in the absence of FGFs growth of the thymus is impaired due to a lack of epithelial cell proliferation. Furthermore, *in vitro* foetal thymic organ culture experiments have shown that NC derived mesenchyme might also be required for the differentiation of lymphoid progenitors into functional T cells (128). These authors have shown that whilst epithelial cells can support the later stages of

T cell development, mesenchymal cells are required for their development beyond the $CD4^-$ $CD8^-$ double negative stage.

The functional importance of NC derived cells during thymus organogenesis has been further suggested by experiments in which their removal or reduction in the pharyngeal region results in greatly reduced size or complete absence of the organ (122). Notably, normal expansion of the epithelial compartment does not occur, if NC derived cells are absent (123). However, it has been reported, based on tissue specific heritable genetic labelling, that once epithelial cells have fully gained the competence to support lymphocyte differentiation, and the vasculature of the thymus is complete, the contribution of NCCs to the thymus is greatly reduced or absent (120, 121). Thus, although it has previously been shown that NC derived cells are critically important for the normal development and function of the thymus, there is controversy regarding their role beyond the early stages of organogenesis.

3.2 Experimental Strategy

To dissect the contribution of NC derived cells in thymus development a tissue specific heritable genetic labelling system was used. Thus, Cre recombinase was expressed under the control of either the *Wnt1* (198) or *Sox10* (199) promoter and regulatory elements, and Cre activity was

reported via activation of a 'silent eYFP', in which the *eYfp* gene preceded by a triple polyadenylation signal flanked by two loxP sites is knocked into the Rosa26 locus (201) (Figure 3.1). As *Wnt1* and *Sox10*, and consequently Cre, are expressed in the neural plate where NCCs originate (113, 199, 205), the latter cells will have the transcriptional stop in front of the *eYfp* gene deleted, thus indelibly marking these cells and their progeny by eYFP expression.

3.3 Results

3.3.1 NC derived cells in foetal and adult mice

In order to determine the pattern of eYFP expression in *Wnt1-Cre;Rosa26*^{eYfp} reporter mice, both embryonic and three month old mice were analysed by stereo and confocal microscopy. At day 13.5 of embryonic development (E13.5) eYFP is expressed in the craniofacial region (Figure 3.2A, left) and in the neurons in the trunk of the embryo (Figure 3.2A, left and right). In the thoracic cavity at E15.5, eYFP expression is detected on the aorta and other arteries derived from the pharyngeal arches (not shown), as well as the thymic rudiment (Figure 3.2B, left). Analysis of E15.5 *Rosa26*^{eYfp} embryos, in which Cre is not expressed under the control of the Wnt1 promoter, showed no eYFP^{+} cells in the thymus indicating specific activation of the reporter only in mice which express Cre (Figure 3.2B, right).

In the three month old mouse, eYFP expressing cells are seen in craniofacial bones and tissues (Figure 3.2C, left), as well as in peripheral nerves (Figure 3.2C, right). Similar patterns of expression were observed in another reporter mouse in which eYFP is also expressed by NC derived cells following Cre expression under the control of NC specific promoter from the *Sox10* gene (not shown). These analyses showed that eYFP expression in tissues and organs from embryonic and three month old *Wnt1-Cre;Rosa26*^{eYfp} mice is restricted to NC derived tissues as previously described (120, 198, 206).

3.3.2 NC derived cells in the foetal and newborn thymus

To follow the kinetics of colonisation of the thymic rudiment by NC derived cells, the pattern of eYFP expression in *Wnt1-Cre;Rosa26*^{eYfp} E13.5, E15.5, E17.5 embryos and newborn mice was examined. At E13.5, some eYFP^{+} cells are present inside the thymus, but most are located at the capsule (Figure 3.3A). At E15.5, eYFP^{+} cells are again found mainly at the capsule, but their frequency is increased inside the thymic rudiment, where they form trabeculae that appear to extend from the capsule into the parenchyma (Figure 3.3B). At E17.5, eYFP^{+} cells are still found at the periphery of the thymus, while those within the organ have organised into a three-dimensional network (Figure 3.3C). Examination of sections from newborn *Wnt1-Cre;Rosa26*^{eYfp} thymi showed that eYFP^{+} cells are still present within

the organ, with many still associated with the capsule (Figure 3.3D). No eYFP^{+} cells were detected in the embryonic or newborn thymus of non-transgenic mice. Thus, NC derived cells enter the thymic rudiment before E13.5, remain there throughout gestation and are still present in the newborn thymus.

3.3.3 NC derived cells in the adult thymus

To determine if cells derived from the NC persist in the thymus beyond birth the distribution of eYFP^{+} cells in three month old *Wnt1-Cre;Rosa26*^{eYfp} thymus was analysed. eYFP expressing cells are readily detectable in the adult thymus at this age, forming a three dimensional network (Figure 3.4, left), with fewer cells found at the capsule than at embryonic stages. High magnification confocal analysis of thymus sections from three month old mice showed that most cells are associated with structures reminiscent of vascular networks (Figure 3.4, right). Measurement of the diameter of vessel-like structures shows that cells expressing eYFP are found in association with vessels ranging from 4.5 to 30μm in diameter, indicating a contribution to both capillaries and larger vessels. This finding suggested that NC derived cells in the adult thymus at three months of age are intimately associated with the blood vasculature.

3.3.4 Migration and redistribution, rather than proliferation, of NC derived cells is responsible for increasing numbers within the thymus rudiment.

During the development of the thymus between E13.5 and E17.5, the number of NC derived cells within the rudiment appears to rapidly increase. This may be due to proliferation of the NC derived cells already within the organ, or continued migration of NCCs from the capsule. To determine if proliferation contributes to thymic colonisation by NCCs during organogenesis, *Wnt1-Cre;Rosa26*^{eYfp} pregnant females were injected once with BrdU, or PBS, at E13.5, E14.5 or E15.5 and the embryos were recovered two days later. It was found that the vast majority of eYFP^{+} cells were BrdU negative, when compared to the extensive incorporation seen in haemopoietic cells. This suggested that the increase of eYFP^{+} cells in the thymus occurs mainly by influx of new NCCs and redistribution within the thymus (Figure 3.5).

3.3.5 NC derived cells are present within the organ and differentiate into perivascular cells before E13.5

NC derived mesenchymal cells have been shown to contribute to connective tissues surrounding the thymic blood vasculature in the chicken embryo (13). However, their precise function in the murine thymus remains controversial.

In order to assess the kinetics of NC derived cell contribution to connective tissue components such as perivascular cells, sections of thymic tissue at different stages of embryogenesis were stained with antibodies recognising markers specific for endothelial cells (endomucin, (207)), pericytes (desmin, (208)) and smooth muscle cells (α-SMA, (209)). Endomucin, thought to be expressed on mature endothelial cells (210), is not detected until E15.5. (Figure 3.6A). Cells expressing this marker appear to be distinct from $eYFP^+$ NC derived cells. As mentioned above, a network of NC derived cells is established in the organ by E13.5, and at this stage, $eYFP^+$ cells also express the pericyte marker, desmin (Figure 3.6B). In contrast, α-SMA expression is not detected until after E15.5, when the formation of the blood vessel network is complete. From this stage onwards $eYFP^+$ cells also start expressing α-SMA (Figure 3.6C).

3.3.6 NC derived perivascular cells are maintained in the thymus into adulthood

It has been suggested that beyond E17.5 in thymus organogenesis, NC derived mesenchymal cells are lost or replaced. However, as shown in Figure 3.4, NC derived cells tagged with eYFP are still detected in the three month old organ. In order to determine the character of the persisting NC derived cells in the adult thymus the expression of markers for blood vessel endothelium, pericytes and smooth muscle cells was examined, as

described above in Figure 3.6, at three months of age. It is shown that eYFP$^+$ cells still surround endomucin expressing endothelial cells in the adult (Figure 3.7A and higher magnification in D) similar to the pattern observed in the embryonic thymus (Figure 3.6A). Since eYFP$^+$ cells appear to surround vessel structures, expression of markers associated with pericytes (desmin and NG2) and smooth muscle cells (α-SMA) were examined. It is shown that some vessel associated eYFP$^+$ cells also express desmin and, in separate stainings, α-SMA (Figure 3.7B & C). Identical patterns of expression were seen for endomucin, desmin and α-SMA using another mouse strain (*Sox10-Cre;Rosa26*^{eYfp}) which also expresses Cre in NC derived cells and thus marks all NC derivatives with eYFP (Figure 3.8A). Furthermore, flow cytometric analyses of *Wnt1-Cre;Rosa26*^{eYfp} and *Sox10-Cre;Rosa26*^{eYfp} (209, 210) thymi show that there is similar expression of the endothelial marker CD31 (0.065% and 2.93%), pericyte marker NG2 (98.8% and 98.5%) and smooth muscle cell marker αSMA (16.2% and 23.4% respectively) in these two reporter lines (Figure 3.8B).

3.3.7 Age-related changes to the NCC contribution to the thymus

As the mouse ages, the perivascular space between the walls of the blood vessels and the surrounding cells in the thymus increases, and is infiltrated with adipose tissue and lymphoid cells (211). This is correlated with a reduction in the export of new T cells into the periphery. In order to assess

whether age-related atrophy is associated with a reduction in NC derived cells, thymi from aged mice were analysed by both immunohistochemical stainings, and flow cytometry. It is shown that at ten months of age, $eYFP^+$ cells are still present within the thymus, and continue to surround blood vessel structures, but there appears to be a reduction in the number of cells that co-express desmin and αSMA (Figure 3.9A). In order to confirm the decrease in the contribution of eYFP cells to the perivascular cell population during thymus organogenesis and ageing thymi from newborn, three and nine month old mice were examined by flow cytometry. Here, the percentage of $CD31^+$, $NG2^+$ and $αSMA^+$ cells which express eYFP was examined. The percentage of $CD31^+$ cells that were also positive for eYFP was consistently low in all time points. It is shown that the percentage of $NG2^+$ cells which express eYFP decreases from 53.6% in the newborn thymus, to 42.5% at three months of age, and to 4.57% by nine months. At birth the population of $αSMA^+$ cells which expresses eYFP is approximately 24.1% of the total thymus. Similar to the results seen by confocal microscopy in Figure 3.9A, there is a decrease in αSMA cells which express eYFP by nine months of age (21.3%) (Figure 3.9B).

Taken together, these results show that NC derived cells, which populate the thymus during organogenesis, persist in the adult thymus having differentiated into at least two different cell types forming part of the same vascular supporting network. However, as the thymus atrophies with age,

there is a reduction in the number of NC derived cells which are associated with the perivascular network. Since NC derived cells persist in the thymus beyond E17.5 and their known role in the early stages of organogenesis, NC derived cells may have additional functions. For example, their differentiation into perivascular cells might affect the development of the thymic blood vasculature.

3.4 Discussion

3.4.1 NC derived cells and early thymus development

Interactions between endothelium and NC mesenchyme initiate thymus development from the third and fourth pharyngeal pouches in the mouse. A number of experiments have shown that, if the contribution from the NC is absent, either by ablation of the neural folds when they form or by enzymatically stripping mesenchyme from the capsule of the E12 thymic rudiment, the thymus is severely hypoplastic or absent (122, 123). It is widely accepted that the main role of NC derived cells in the thymus occurs during early organogenesis, where they stimulate the proliferation and maturation of epithelial cells through FGF signalling (108). This conclusion was based on experiments that showed that thymic lobes devoid of mesenchyme did not grow and become lobulated in culture, and there was a three fold decrease in BrdU incorporation in the epithelial cell compartment

(124). Epithelial cells are required for the maturation of T lymphocyte progenitors into naïve single positive $CD4^+$ or $CD8^+$ T cells. Thus, defects in NC mesenchyme also have an impact on T cell output from the thymus. Integrin family members, such as integrin α4, α5, αV, β1 and β3, were demonstrated to be expressed on the surface of NC derived mesenchymal cells present at the capsule of the thymus (121). Thus, a further role in attraction of T lymphoid progenitors into the rudiment before it is vascularised, was also suggested.

Chick-quail chimeras first demonstrated a contribution of NC derived cells to the connective tissues that surround blood vessels within the thymus (13). These experiments were later confirmed with lineage-tracing tools, in which all NC derived cells were marked by LacZ expression under the control of Wnt1 or P0 promoter and regulatory elements (120, 121). These analyses showed that NC derived cells are associated predominantly with the capsule of the rudiment from E11.5 until E17.5, but are lost or replaced thereafter. This led to the hypothesis that NC derived cells were critical to the initial formation of the thymus, but once epithelial cells have fully gained the competence to support thymocyte development and the vasculature is complete, they are no longer required.

The data presented here shows a continued contribution of NC derived cells in the newborn and adult thymus, even persisting up to nine to ten months of

age. These data appear to contradict previous studies, but can be explained by differences in the experimental approach. Jiang (120), Yamazaki (121) and their colleagues, expressed Cre recombinase under the Wnt1 or P0 promoter and regulatory elements. Cre recombinase was used to drive LacZ expression from the Rosa26 locus. Here, a similar strategy was used, using eYFP knocked into the Rosa26 locus as a marker of NC derived cells, rather than LacZ. The apparent difference in expression or eYFP and LacZ, despite being under the control of the same promoter, may be due to the high CpG content in the *LacZ* gene and thus a predisposition to methylation induced silencing with age (212). $eYFP^{+}$ cells are detected in the three month old thymus with identical distribution (around vessels) and characteristics (expressing desmin/NG2 and α-SMA) using two independent mouse lines (*Wnt1-Cre* and *Sox10-Cre*) each expressing Cre in a NCC pattern. This makes it unlikely that eYFP expression is due to identical ectopic activation of the *Cre* gene.

The indication that the $Rosa26^{LacZ}$ reporter may be silenced highlights just one of the caveats of lineage tracing tools. Other limitations of these tools include ectopic expression of Cre on other lineages, inefficient Cre-mediated excision and incomplete penetrance (Williams, A. PhD Thesis 2007 UCL). In this report Cre expressed under the control of the Wnt1 promoter was used. Wnt1 is expressed in the neural plate, dorsal neural tube, and an early migratory neural crest population. It is extinguished as cells migrate away

from the neural tube and is not expressed again at any other point in development or in the adult. The mice used in this report were generated by insertion of Cre recombinase into the endogenous locus, and thus, is less likely to experience position effect variegation. When crossed to Rosa26^{eYFP} reporter mice, eYFP^{+} cells are present in an identical pattern to that expected of NCCs, thus there is expression of the reporter in the craniofacial region, in peripheral neurons and in the embryonic thymus (251, 221). Moreover, the pattern of expression seen in the Wnt1-Cre;Rosa26^{eYFP} mice was confirmed with another mouse line, Sox10-Cre;Rosa26^{eYFP}. Thus it is unlikely that silencing, inefficient deletion or incomplete penetrance affects both of these Cre expressing lines.

The experiments that used LacZ to mark NC derived cells indicate that they contributed mostly to the capsule, with only a few located in the parenchyma. However, earlier reports using chick-quail chimeras showed that NC derived cells contribute to connective tissues and surrounded the blood vessels throughout the rudiment. These data are in support of the latter experiments, since NC derived cells were found throughout the organ from E13.5 to adults. Moreover, using a marker of proliferation, BrdU, it is shown for the first time that very few NC derived cells are proliferating between E15.5 and E17.5, thus favouring a mechanism by which migration and redistribution in the organ contributes to the formation of a three dimensional network.

3.4.2 NCCs differentiate into perivascular cells

NCCs contribute to most of the connective tissues in the head of vertebrates, including, but not limited to, neurons and glia of the autonomic nervous system, tendons, smooth muscle, chondrocytes, osteocytes and melanocytes and the perivascular mesenchyme of the embryonic chick thymus (13). In addition, all aortic arch-derived vessels and capillaries that supply the forebrain are ensheathed with pericytes of NC origin, while capillaries in the rest of the central nervous system are associated with pericytes derived from mesoderm (111). Thus, there is a clear precedent for NC differentiation into perivascular cells elsewhere in the embryo, including the embryonic thymus. Our data, and a recent report by Muller and colleagues (213), using a similar lineage-tracing tool, showed that NC derived cells were not lost from the thymus and continued to be found in the adult organ.

Contribution to the perivascular network of cells in the thymus indicates a further possible role for NC derived cells in organogenesis than previously described. Thus, NC derived cells may direct the formation and/or function of the specialised thymic blood vasculature, which has a double-walled morphology and constitutes a blood-thymus barrier. The function of the blood-thymus barrier is to prevent developing thymocytes in the thymic cortex from being exposed to antigen circulating in the blood.

Data is presented here showing that NC derived perivascular cells are located both at the capsule and within the parenchyma at E13.5. Blood vessels are postulated to form in the thymus later, at E14, from endothelial sprouts which branch off vessels nearby the capsule. Since NC derived perivascular cells are already present throughout the rudiment, they may play a role in attracting vessels into the parenchyma. To examine such a possibility, one would need to examine thymus vascularisation in the absence of NC derived cell contribution. Alternatively, NC differentiation into perivascular cells may require signals from endothelial cells. If the latter possibility is correct, then NC differentiation would be blocked in the absence of thymic blood vessels. The question, as to the role in NC derived cell in blood vessel development, will be explored in the next chapter.

NC derived perivascular cells are eventually replaced by cells of other mesenchymal origin and it is intriguing that this takes place at the same time as thymic involution. Since NC derived cells have not been demonstrated to persist in the adult thymus until now, the fate of these cells in the aged thymus is unknown. Studies examining perivascular turnover in rodents and humans are rare, but one such report gave the estimate that 30% of perivascular cells were replaced during a three-month period in rats (214). Since NC derived cells may be replaced, a number of questions arise, such as; What is the origin of the cells that replace them? Does the change in

origin affect their function, and/or the process of thymus involution? To address these questions, thorough analyses of the kinetics of NC derived perivascular cell loss are required. Moreover, it will be necessary to trace the origin of the replacing cells, and assess their function in comparison to NC derived perivascular cells. There are a number of possible ways to do this, including looking at the expression profile of genes known to be required for vessel function or adhesion in both subsets of cells by microarrays, or assessing their proliferation and survival *in vitro*.

3.4.3 What happens to NC derived cells in the aged thymus?

Gradual replacement of NC derived perivascular cells in aged mice by cells of other mesenchymal origin may contribute to the mechanism of thymic involution. Involution results in a gradual loss of structure and progressive increase in adipocytes, disruption of endothelial vessel walls, a decrease in lymphoid progenitors and epithelial cell populations causing a decline in thymic output (215, 216). NC derived cells have not previously been reported in the aged thymus, and thus, their role in involution is unknown. Loss of NC derived cells might contribute to involution by disrupting the perivascular network of cells that supports the vasculature. Moreover, since NC derived cells are known to play a crucial role in epithelial cell proliferation and maturation, their loss may also affect epithelial-thymocyte interactions. If this is the case, then re-generation of the NC derived cell compartment may be

an attractive candidate for further research. Interestingly, NC stem cells (NCSCs) are known to exist in other adult tissues, albeit with reduced potential to form different types of cells, and thus may also be present in the adult thymus.

NCSCs have been demonstrated to exist in at least two different tissues; the enteric nervous system of the gut and whisker follicles of the facial skin (217-219). Gut derived NCSC progenitors, for example, have been demonstrated to persist in the gut as late as postnatal day 110 in the rat (217), but their expression of cell surface markers and ability to self renew and differentiate decreases with age (217). Moreover, when NCSCs from gut or sciatic nerve were transplanted into the NC migratory stream of the chick embryo *in vivo*, gut NCSCs gave rise primarily to neurons, while sciatic nerve NCSCs gave rise exclusively to glial cells (220). Thymus-resident NCSCs may have a similar limited potential that decreases further with age.

The self-renewal capacity of NC derived cells from the aged thymus could be assessed *in vitro* by neurosphere assay. This method is based on the dogma that stem cells and their progeny can be stimulated to proliferate when they are exposed to certain growth factors (221). When plated at appropriate densities, continued cell division generates a non-adherent spherical cluster of cells, called a neurosphere (222, 223). They can be isolated, dissociated and re-plated to form secondary spheres, however, to ensure they are *bona*

fide stem cells they must demonstrate self-renewal over an extended period of time and generate a large number of progeny (224). If NCSCs exist in the adult thymus, they could be stimulated to provide new perivascular cells and induce re-generation of the epithelium, thus reversing age-related involution.

3.4.4 Summary

The contribution of NC derived cells to the embryonic and adult thymus was analysed using a genetic lineage-tracing tool. It was found that NC derived cells were not lost from the organ in late gestation as previously described. In contrast, here it is shown that they differentiate into perivascular cells and continue to be present up to nine to ten months of age. Thus, NC derived cells may have an additional role in thymic blood vessel formation or function, in addition to their roles in early organogenesis.

Figure 1: Wnt1 and Sox10 Cre expression, and ROSA26 reporter transgenes. (A) Schematic diagram of the Wnt1-Cre transgene (Adapted from 198). Top; untargeted locus. Bottom; Transgene expressing Cre. Black and white boxes represent, respectively, translated and untranslated parts of *Wnt1* exons. **(B)** Sox10-Cre reporter transgene (Adapted from (199) and (303)). Top; untargeted locus. Bottom; Transgene expressing Cre. Black and white boxes represent, respectively, translated and untranslated parts of *Wnt1* exons. **(C)** Structure of the untargeted *ROSA26* locus (Top), the targeted locus (Middle) and the targeted locus after Cre-mediated excision of the *loxP*-flanked (*PGK-neo*, tpA) cassette (bottom). *LoxP* sites are indicated by solid arrowheads (Adapted from 149).

Figure 2: Expression of eYFP in fetal and adult *Wnt1-Cre;Rosa26eYfp* mice.

(A) Whole-mount E13.5 *Wnt1-Cre;Rosa26eYfp* embryos were fixed, stained with an antibody recognizing eYFP, optically cleared with BABB and analyzed by confocal microscopy. Serial optical sections of the whole embryo were captured and reconstituted to form a three-dimensional rendering. White arrow: Craniofacial structures. Red arrow: Trunk neurons. **(B)** E15.5 thymi from *Wnt1-Cre;Rosa26eYfp* (Left) and wild-type littermate control thymi (right) were microdissected, and analyzed by stereo microscopy. Dotted grey line indicates outlines of wild-type thymus lobes. Arrow: capsule of thymic lobe. **(C)** Left: Location of eYFP+ cells in the craniofacial region was determined in three month old *Wnt1-Cre;Rosa26eYfp* mice by stereo microscopy. White arrow: Facial region. Right: Mice were further dissected to reveal expression of eYFP on peripheral nerves. White arrow: Axons. Results are indicative of four experiments consisting of twelve mice.

Figure 3: Expression of eYFP in thymi from E13.5, E15.5, E17.5 and newborn *Wnt1-Cre;Rosa26eYfp* mice. Whole-mount E13.5, E15.5, E17.5 and Newborn *Wnt1-Cre;Rosa26eYfp* (left) and control *Rosa26eYfp* (right) embryos were fixed, stained with an antibody recognizing eYFP, optically cleared with BABB and analyzed by confocal microscopy. Serial optical sections of the whole embryo were captured and reconstituted to form a three-dimensional rendering.

Figure 4: Expression of eYFP in adult *Wnt1-Cre;Rosa26eYfp* thymus. Whole-mount three month old adult *Wnt1-Cre;Rosa26eYfp* thymi were fixed, stained with an antibody recognizing eYFP, optically cleared with BABB and analyzed by confocal microscopy. Left; low magnification, right; high magnification. White arrow; capsule. Red arrow; vessel-like structure.

Figure 5: NCCs enter the thymus by migration and redistribution, rather than proliferation. E15.5, E16.5 and E17.5 *Wnt1-Cre;Rosa26eYfp* embryos were taken from pregnant female mice that had been injected with one dose of BrdU two days earlier (66 micrograms per gram of mouse). Thymic rudiments were dissected from the embryos, processed as previously described and stained with antibodies against BrdU (red) and eYFP (green). Sections were optically cleared with BABB and analyzed by confocal microscopy. White arrows: capsule of thymic lobe.

Figure 6: Development of blood vessel and eYFP+ networks in embryonic thymus. (A, B, & C) Thymi from *Wnt1-Cre;Rosa26eYfp* embryos were dissected at E13.5 (left column), E15.5 (middle column) and E17.5 (right column), processed as previously described and stained with antibodies recognizing eYFP (green), endomucin (red) **(A)**, desmin (red) **(B)** and α-SMA (red) **(C)**, and analyzed by confocal microscopy. White arrows: capsule of thymic lobe. Red arrows: Vessels.

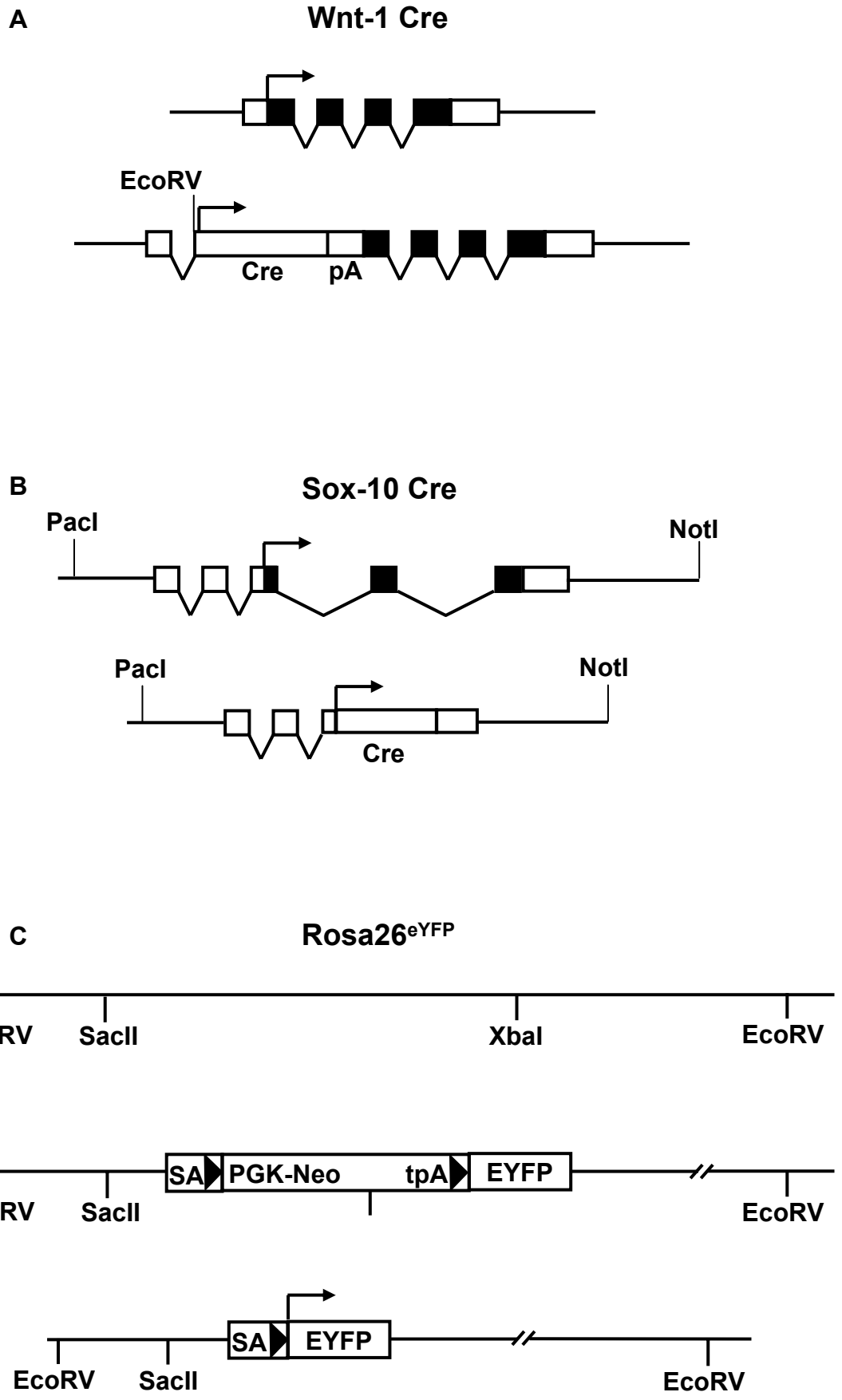
Figure 7: Differentiation of eYFP^{+} cells in the adult thymus. (A, B & C) Thymi from three month old *Wnt1-Cre;Rosa26*^{eYfp} mice were dissected, processed as previously described and stained with antibodies against eYFP (green) and **(A)** endomucin (red), **(B)** desmin (red) and **(C)** α-SMA (red), and analyzed by confocal microscopy. White arrows: capsule of thymic lobe. Red arrow: Vessels. **(D)** Left; middle panel of **(A)** with box highlighting magnified section, right.

Figure 8: Analysis of *Sox10-Cre;Rosa26eYfp* mice confirms the pattern of eYFP expression seen in *Wnt1-Cre;Rosa26eYfp* mice is specific to NCCs. **(A)** Thymi from three month old *Sox10-Cre;Rosa26eYfp* mice were dissected, processed as previously described and stained with antibodies against eYFP (green), endomucin (red, left) and α-SMA (red, right). White arrows: capsule of thymic lobe. Red arrow: Vessels. **(B)** Thymi from three month old *Wnt1-Cre;Rosa26eYfp* (top) and six week old *Sox10-Cre;Rosa26eYfp* (bottom) mice were digested with collagenase, stained with markers recognizing endothelial cells (CD31), pericytes (NG2) and smooth muscle cells (αSMA), and analyzed by flow cytometry. Cells were gated on eYFP+ expression and the percentages of CD31+, NG2+ and αSMA+ cells were calculated.

Genotype and ID of mouse	% CD31	% NG2	% αSMA
Wnt1CreR26ReYFP	0.065	98.8	16.2
	0.12	94.1	20.8
	0.054	97.8	17.6
Sox10Cre-R26ReYFP	2.93	98.5	23.4
	0.53	96.8	20.6
	1.87	97.9	19.8

Figure 9: eYFP+ cells within the aged thymus. (A) Thymi from *Wnt1-Cre;Rosa26eYfp* mice at 10 months of age were stained with antibodies recognizing eYFP (green) and endomucin (left), desmin (middle) and αSMA (right). Red arrows: vessels. **(B)** Thymi from *Wnt1-Cre;Rosa26eYfp* mice at birth, three months and nine months of age were collagenase digested and stained with antibodies recognizing endothelial cells (CD31), pericytes (NG2) and smooth muscle cells (αSMA). The resulting single cell suspension was first gated on CD31, NG2 and αSMA expressing cells, and then the percentages of eYFP+ cells within these populations was determined.

Age and ID of mouse	% YFP (of CD31+)	% YFP (of NG2+)	% YFP (of αSMA+)
Newborn	0	53.6	24.1
	0.01	62.5	28.35
	0	53.2	22.9
Three months old	0.24	42.5	30.2
	0.12	56.4	35.2
	0.01	49.1	32.8
Nine months old	0	4.57	21.3
	0.18	10.9	23.5
	0.05	5.89	19.8
Littermate control	0	0	0
	0	0.1	0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NotI
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SacII
XbaI
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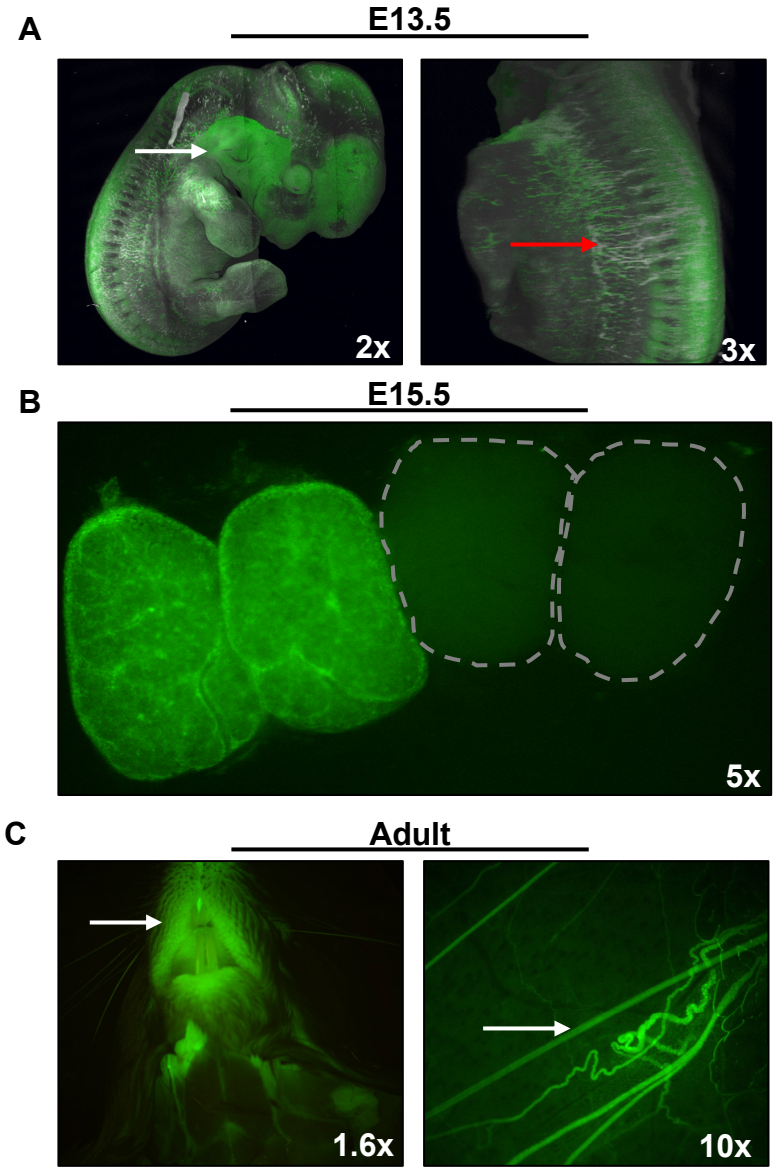
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C
Adult
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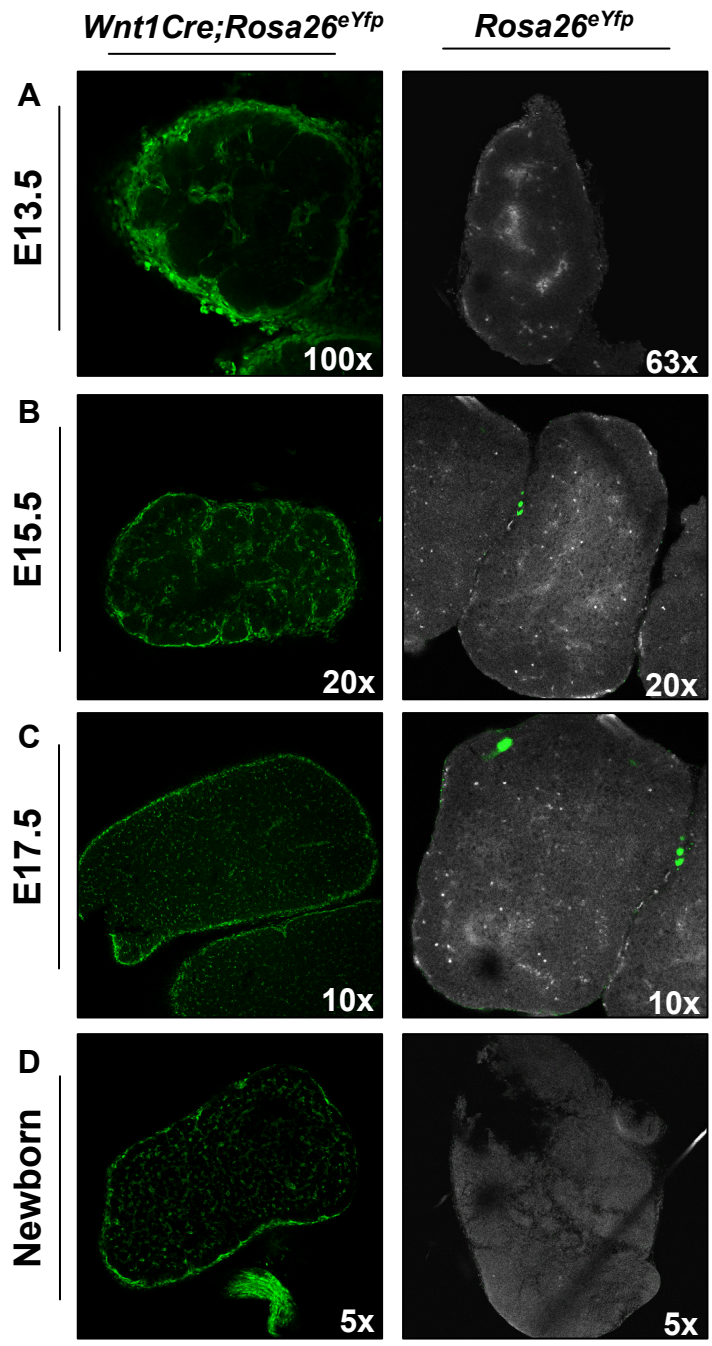
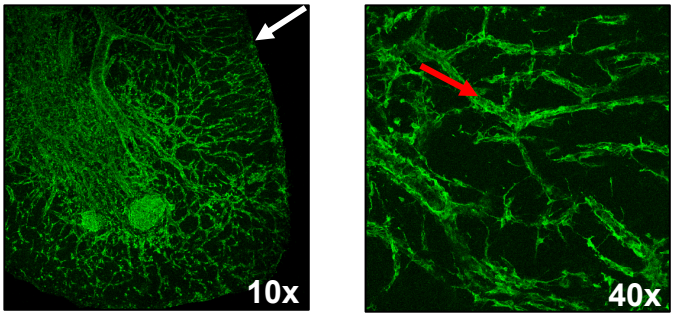
10x



YFP Background autofluorescence

Adult



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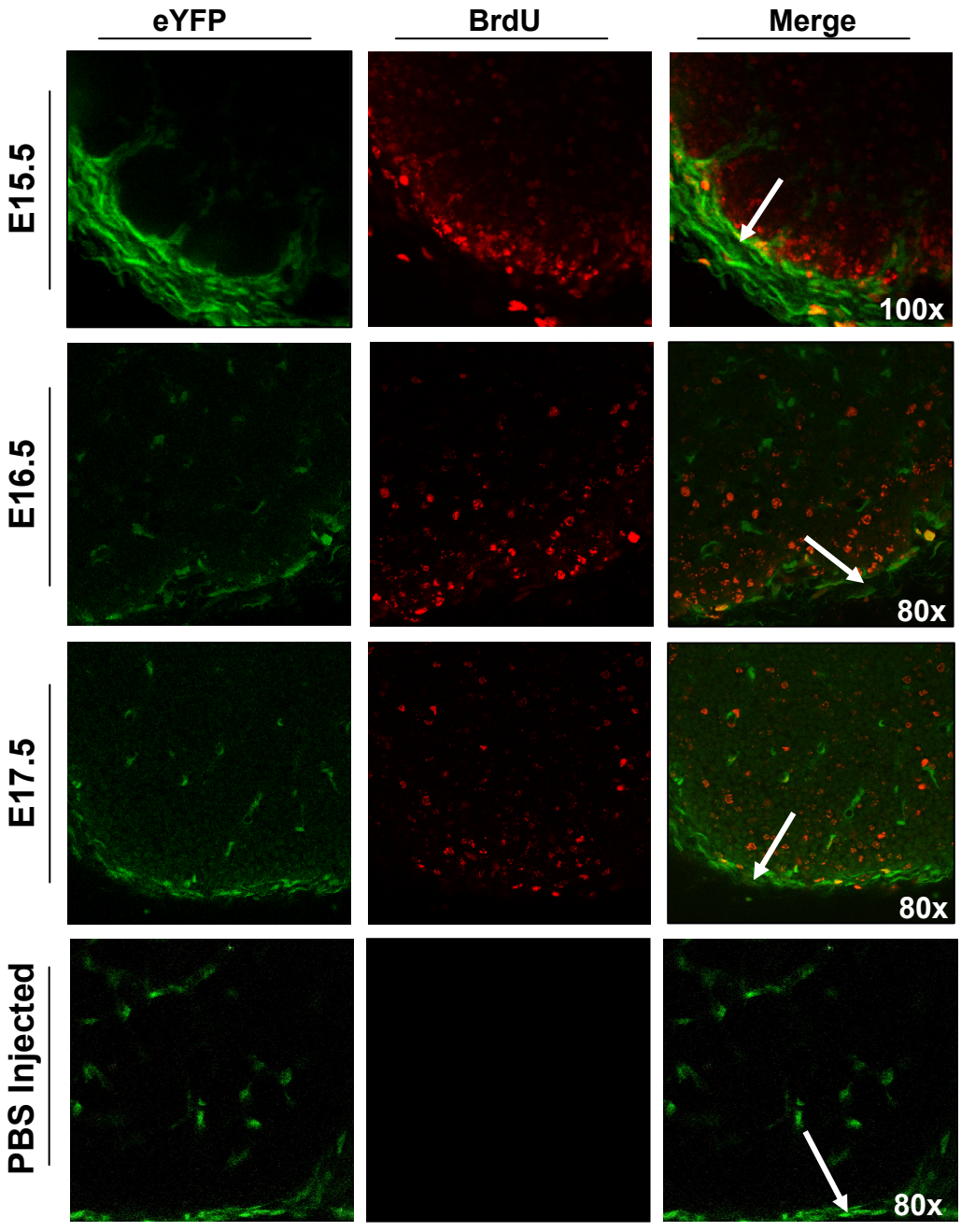
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E16.5
E17.5
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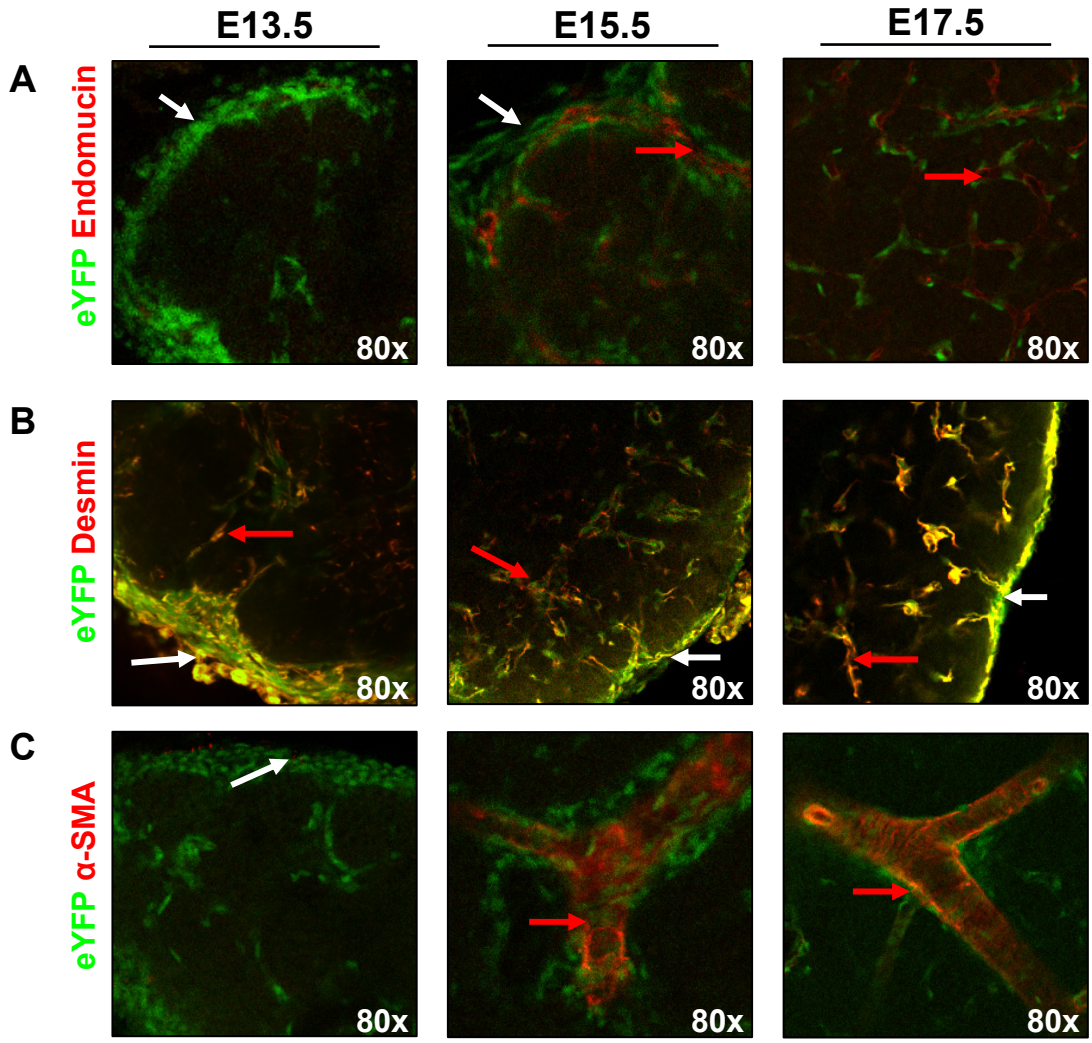
80x
80x
80x


E13.5
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E17.5
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eYFP Endomucin
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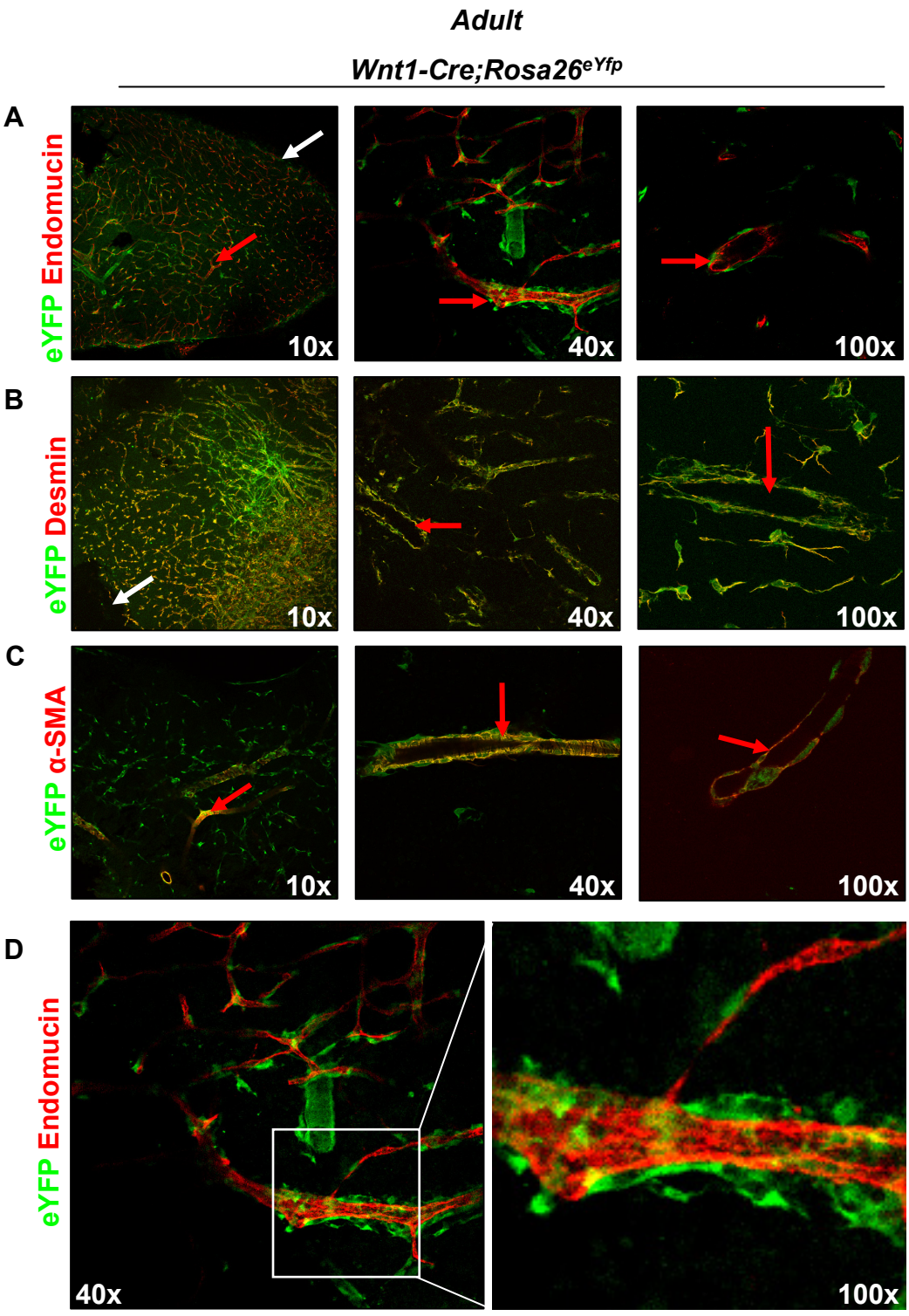
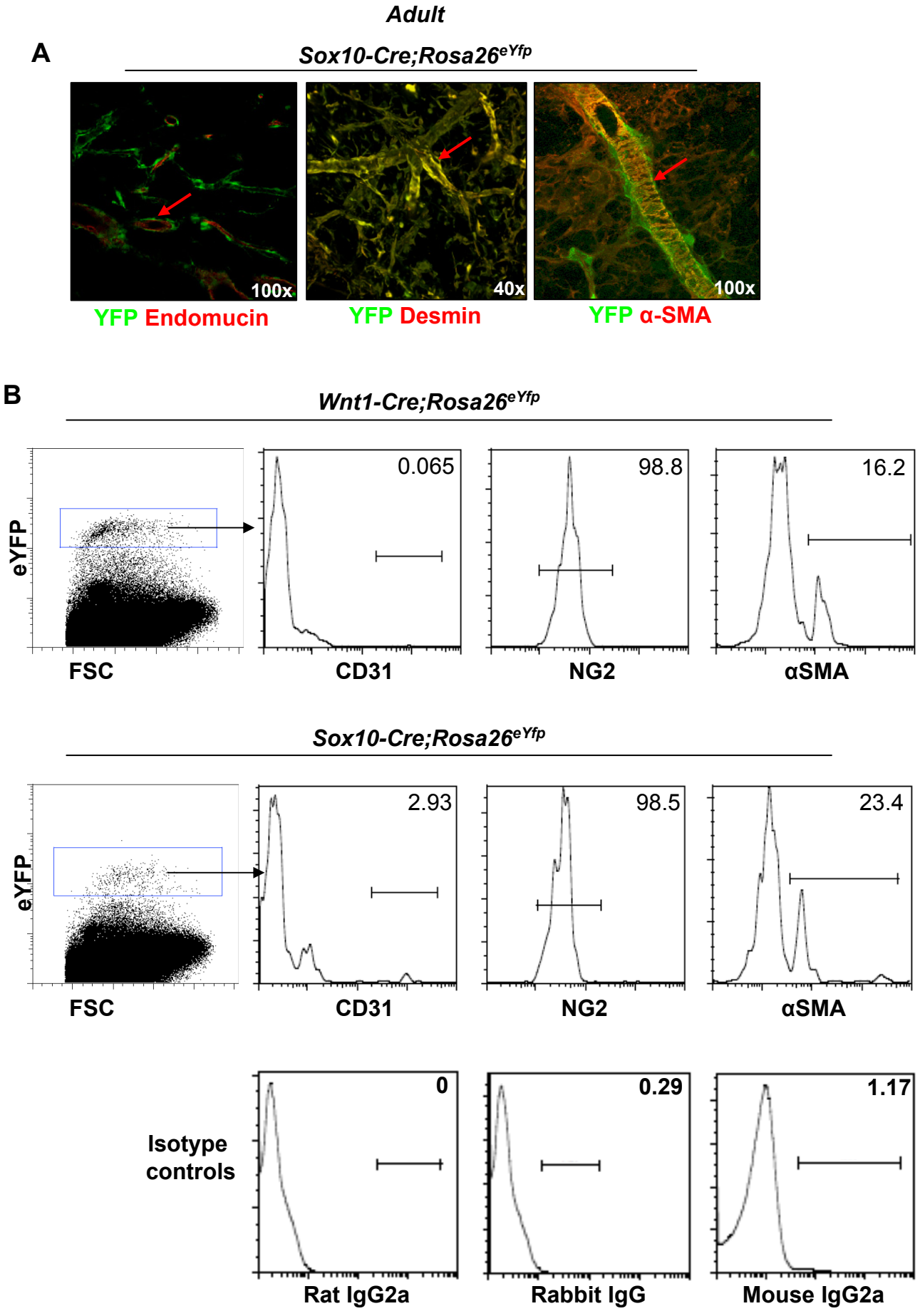

Adult
Wnt1-Cre;Rosa26eYfp
A
eYFP Endomucin
10x
40x
100x
B
eYFP Desmin
10x
40x
100x
C
eYFP α-SMA
10x
40x
100x
D
eYFP Endomucin
40x
100x


Adult
A
Sox10-Cre;Rosa26eYfp
100x
40x
100x
YFP Endomucin
YFP Desmin
YFP α-SMA
B
Wnt1-Cre;Rosa26eYfp
eYFP
FSC
0.065
CD31
98.8
NG2
16.2
αSMA
Sox10-Cre;Rosa26eYfp
eYFP
FSC
2.93
CD31
98.5
NG2
23.4
αSMA
Isotype controls
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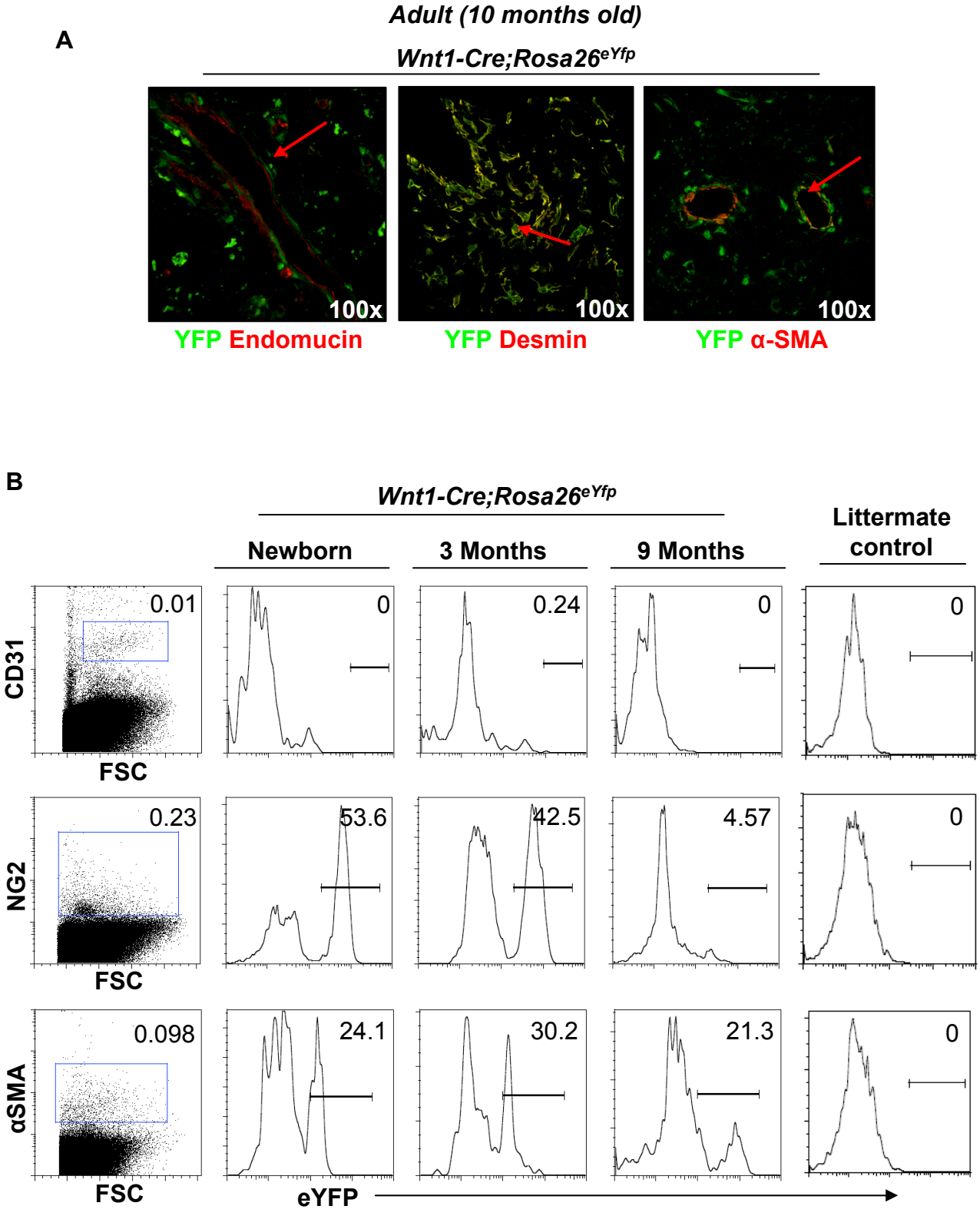
Rat IgG2a
0.29
Rabbit IgG
1.17
Mouse IgG2a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Adult (10 months old)
Wnt1-Cre;Rosa26eYfp
100x
100x
100x
YFP Endomucin
YFP Desmin
YFP α-SMA
B
Wnt1-Cre;Rosa26eYfp
Newborn
3 Months
9 Months
Littermate control
CD31
NG2
αSMA
FSC
0.01
0
0.24
0
0
0.23
53.6
42.5
4.57
0
0.098
24.1
30.2
21.3
0
eYFP

Chapter Four: Do NC derived cells play a role in thymic blood vessel development?

Vascularisation of the thymus is thought to occur by branching inwards of vessels near the capsule at E14. In contrast, here it is shown that endothelial precursor cells are already present within the thymus at E13.5. Thus, it is possible that vessel structures form within the thymus first, or in parallel, and then connect with vessels that branch inwards from the capsule. NC derived mesenchymal cells express both PDGFRα and β receptors during thymus development in the embryonic period, but downregulate expression of the α receptor thereafter. Thus, the change in receptor expression may correlate with a change in function of NC derived cells, from stimulating the proliferation of epithelial cells to supporting the thymic blood vasculature.

4.1 Experimental Rationale

Results from the previous chapter showing that NCCs differentiate into perivascular cells indicate a possible role in thymic blood vessel formation and/or function. Blood vessels are postulated to form in the thymus at E14 by branching inwards of epithelial sprouts from nearby vessels. As vascularisation is a process resulting from the interaction of different types of cells, and since it was shown here that NC derived perivascular cells were already within the thymic rudiment by E13.5, it was possible that they have a role in the formation of vessel structures.

Endothelial sprouts are formed by proliferation of endothelial cells from existing vessels, and migrate into the thymus in response to angiogenic stimuli such as vascular endothelial growth factor (VEGF) (43). VEGF, produced by thymic epithelial cells and mesenchymal cells (44), is an endothelial cell mitogen and a permeability enhancing factor that stimulates angiogenesis (45). Recently, it has been demonstrated that pericytes are an important source of VEGF and may migrate ahead of endothelial cells guiding the sprouting processes (46). Endothelial cells on the other hand produce PDGF-B that in turn attracts PDGFRβ^{+} perivascular mesenchyme to the walls of growing blood vessels (48). Perivascular cells, such as pericytes and smooth muscle cells, then wrap around the vessels providing structural

support and regulating endothelial cell function. In the absence of perivascular cells or PDGFRβ signalling, vessel structures are abnormal and prone to haemorrhage (49). Indeed, this is a characteristic of pathological states such as oedema and diabetic retinopathy, where vessels appear abnormal and fragile (50).

Of particular importance to blood vessel development are the platelet derived growth factor (PDGF) family of molecules. PDGF ligands are composed of a disulphide-bonded dimer of two polypeptide chains, A, B, C or D, either as homodimers (AA, BB, CC or DD) or heterodimers (AB only). Their receptors, PDGFRs, are protein tyrosine kinases and consist of two chains, α and β. The α receptor (αR) can bind to both PDGF-A and PDGF-B, whereas the β receptor (βR) binds only PDGF-B with high affinity (for review, see (225)). Ligand binding induces the receptors to dimerise as αα, ββ, or αβ pairs, resulting in a conformational change that activates the kinase domain and a downstream signalling cascade involving molecules that interact with the intracellular domain of the receptor.

PDGFRα expression is required for the development of craniofacial structures and the aortic arch. Mice with the *Ph* deletion, a naturally occurring deletion of the gene encoding the PDGFRα receptor, have thymic defects ranging from complete absence of the organ to the diminished size of one or both lobes (226). In contrast, PDGFRα deletion specifically on

NCCs is less severe in terms of thymus development, and a decrease in organ size is seen in only a few embryos (227). Depletion of PDGFRα^{+} mesenchyme from embryonic thymi prior to their transplantation to ectopic sites results in the formation of functional yet hypoplastic thymic tissue. Thus, PDGFRα is clearly required at some level for the development of the thymus. In contrast, the role of PDGFRβ in thymus development is unknown. Broad deletion of the β receptor is neonatal lethal due to extensive haemorrhaging, oedema and lack of kidney mesangial cells (204). These defects were attributed to a compromise in vessel integrity where perivascular cells do not adhere to vessel walls correctly.

Since NCCs differentiate into perivascular cells, which are important for remodelling of the vasculature by expressing, amongst others, PDGFRs, the role of this signalling axis was investigated during embryonic thymic blood vessel development.

4.2 Experimental Strategy

A. PDGFRβ$^{-/-}$ knock out mice

In order to determine whether PDGFRβ signalling has a role in thymus development embryos lacking this receptor were analysed. PDGFRβ$^{-/-}$ mice were generated by replacing parts of exon 1 and 2 with a promoterless β-

geo gene in the PDGFRβ locus by homologous recombination, creating a null allele (204). The structure of the blood vasculature was assessed by staining with endothelial and perivascular markers, and imaged by confocal microscopy.

B. Inhibition of thymic vascularisation

Endothelial cells may appear before NC derived cells within the developing rudiment, and thus it is possible that they induce the differentiation of NCCs into perivascular cells. In order to investigate this possibility, vessel development was blocked in foetal thymic organ culture (FTOC) and the contribution to, and differentiation of, NC derived cells within the rudiments were assessed. For this purpose, a biochemical inhibitor and a monoclonal antibody that have been demonstrated to inhibit blood vessel formation were used. Sunitinib Malate, an anti-angiogenic inhibitor, has been approved for the treatment of certain cancers. It mediates its affects by targeting multiple receptor tyrosine kinases (RTKs), including PDGFRs, VEGFs and KIT resulting in reduced vascularisation. It also targets FLT3, a receptor tyrosine kinase, by inhibiting its phosphorylation and thereby inhibiting proliferation and inducing apoptosis (228-231). DC101 is an anti-angiogenic monoclonal antibody specific for one of the VEGF receptors, VEGFR-2, (encoded by FLK1 in mice and KDR in humans). DC101 application to tumour bearing mice first stabilises tumour vessel structures by increasing pericyte

coverage. Stabilisation of vessels is followed by pericyte loss and decreased basement membrane thickness, vessel diameter and density. The physiological effect is a decrease in microvessels, increased tumour cell apoptosis, decreased tumour cell proliferation and extensive tumour necrosis (232, 233).

C. Ablation of NC derived cells in mouse embryos

It is equally possible that NC derived cells are present within the rudiment first, and direct the formation of the thymic blood vasculature. To determine whether NC derived cells were required for the formation of the blood vessels in the thymus, mice were generated in which these cells were ablated. To this end, *Wnt1-Cre;Rosa26*^{eYfp} mice were crossed to DIP-R1 mice, which carry a conditional expression construct for diphtheria toxin. Diphtheria toxin is secreted from *Corynebacterium diphtheriae* and cleaved by the protease furin, generating two fragments; DT-A and DT-B (234, 235). The DT-A fragment is extremely toxic, a single molecule of the protein is sufficient to induce cell death, whereas DT-B is responsible for cell surface binding and translocation of the DT-A fragment into the cytosol (236). An expression construct was generated in which a transcriptional stop signal flanked by LoxP sites upstream of the DT-A gene, and was inserted into the ubiquitously expressed Rosa26 locus. Transcription of the cDNA is prevented by the presence of the upstream triple polyadenylation signal, but

upon Cre expression under the control of the Wnt1 promoter, the stop signal is excised and DT-A is expressed (Williams A, Ph.D Thesis 2007). However, due to the extensive role of NCCs in formation of craniofacial structures and heart development, these embryos died at E12 and it was not possible to examine the vascularisation of the thymus in these mice.

4.3 Results

4.3.1 NC derived cells in the thymus express PDGF receptors

PDGF receptor expression is characteristic of mesenchymal cells and binding of PDGF mitogens directs a number of processes including gene regulation and cell cycle control, and plays a crucial role in blood vessel development. To determine which cellular compartments express PDGF receptors in the thymus, the expression of PDGFRα and β was assessed on perivascular ($NG2^+$), endothelial ($CD31^+$), haematopoietic ($CD45^+$) and epithelial ($CD45^-$ MHC Class II^+) cell populations in thymi from three month old C57BL/6 mice. $PDGFR\alpha^+$ cells can be fractionated into different cell lineages; 73.9% of perivascular cells (NG2); 11.7% endothelial cells (CD31); 9.05% haematopoietic cells (CD45) and 6.6% thymic epithelial cells (MHC Class II). PDGFRβ has a similar pattern of expression; 52.6% perivascular cells (NG2); 10% endothelial cells (CD31); 15.5% hematopoietic cells (CD45) and 18.7% thymic epithelial cells (MHC Class II) (Figure 4.1). Thus,

PDGFRα and β are preferentially expressed by cells of mesenchymal nature including pericytes.

In order to determine whether NC derived cells are also involved in the PDGFR/PDGF axis of signalling during thymic development, the expression of PDGFRα and PDGFRβ on thymic eYFP^{+} cells, from *Wnt1-Cre;Rosa26*^{eYfp} embryos and adult mice, were studied by flow cytometry. In one such analysis of E15.5 thymic rudiments, 63.1% of eYFP^{+} cells express PDGFRα and 92.8% express PDGFRβ (Figure 4.2A, top two panels). In contrast, in the three month old thymus the majority (97.7%) of eYFP^{+} cells still express PDGFRβ, but PDGFRα was found only on 9.2% of the cells (Figure 4.2A, bottom two panels). This was confirmed by confocal microscopy which showed that PDGFRα is expressed only by eYFP^{+} cells found in the capsule of the three month old thymus, whereas PDGFRβ was expressed by eYFP^{+} cells found both at the capsule and within the organ (Figure 4.2B).

4.3.2 Thymus development is normal in PDGFRβ$^{-/-}$ mice

Mutations which knockout the function of PDGFRα often result in hypoplastic but functional thymi (49, 204), however the role of PDGFRβ signalling in the thymus is unknown. It has been shown that PDGFRβ is required for recruitment of pericytes to the walls and subsequent blood vessel stabilisation. Indeed, embryos deficient in PDGFRβ do not survive beyond

birth due to sudden microvascular haemorrhaging and oedema (49, 204). Here, it is shown that in both the embryonic and three month old thymus, NC derived cells are PDGFRβ^{+} (Figure 4.2A), suggesting a mechanism by which NC derived cells are recruited to the blood vasculature. However, by examining markers specific for pericytes and endothelial cells in E15.5 and E18.5 PDGFRβ deficient embryos, it is shown here that in the absence of this signalling receptor the thymic blood vasculature appears to have developed normally (Figure 4.3).

4.3.3 Co-development of blood vasculature and NC derived cell networks

It is thought that the thymus becomes vascularised at E14.5 whereby angiogenic growth factors produced in the thymus activate growth factor receptors on endothelial cells present on blood vessels near the developing thymus anlagen (43). The activated endothelial cells release proteases which degrade the basement membrane, allowing endothelial cells to escape the original vessel wall and proliferate into the surrounding matrix and parenchyma of the organ, forming sprouts. Since NC derived cells of perivascular character are present within the thymus before E13.5 (Figure 3.6), it is possible that these cells are the source of angiogenic growth factors and thus direct the development of the blood vasculature. In order to determine which develops first, the blood vasculature or the NC derived

perivascular cell network, the expression of the early endothelial marker, CD31 was examined. At E13.5, $CD31^+$ cells were already present throughout the thymic rudiment indicating that endothelial cells, or endothelial precursor cells, are already present at this stage (Figure 4.4) (This experiment was performed by Julie Sheridan, University of Edinburgh). Together these data indicate that the thymic blood vasculature may form from thymus-resident endothelial stem cells, or angioblasts, which proliferate into de novo endothelial cell structures that connect up with vessels at the periphery, rather than from endothelial sprouts from vessels nearby the capsule that migrate into and colonise the rudiment.

4.3.4 Blocking blood vessel formation in the thymus may disrupt the development of the NC derived perivascular network

Since both NC derived perivascular cells and endothelial precursor cells are present within the rudiment at E13.5, it remains unclear which network develops first and, thus, whether the development of one network influences that of the other. It was not possible to determine whether ablating the NCC component of the embryonic thymi perturbs vascularisation of the thymus, due to early death of *Wnt1-Cre;Rosa26*^{eYFP}*;DIP-R1* embryos. However, it was possible to assess the contribution and differentiation of NCCs in the absence of blood vessel development using a VEGFR-2 blocking DC101 antibody, or in other cultures, Sunitinib Malate, a receptor tyrosine kinase

inhibitor. The cultured foetal thymi were fixed and stained with blood vessel markers after four days incubation with inhibitor, and analysed by confocal microscopy.

Incubating foetal thymi with DC101 antibody for four days does not appear to affect the development of thymic blood vessels or their density (Figure 4.5, left). Moreover, NC derived cells were still associated with endomucin^{+} endothelial cells, similar to those seen in foetal thymi cultured with a control antibody, biotinylated anti-human CD2 (IgG1) (Figure 4.5, right). However, incubation with Sunitinib Malate for four days resulted in a marked absence of blood vessel development, with only clumps of endothelial cells observed. In these cultures, NC derived cells did not form their usual three dimensional network of cells throughout the parenchyma (Figure 4.6A, left), compared with thymi incubated without inhibitor (Figure 4.6A, right). However, the remaining NC derived cells, mostly located at the capsule, continued to express the perivascular marker desmin (Figure 4.6B). These results indicate that the development of a three dimensional NC derived cell network throughout the rudiment was perturbed in the absence of blood vessel formation. Nevertheless, differentiation of NCCs into perivascular cells did occur indicating that this process may be independent of vessel formation. However, since this inhibitor blocks several other receptor tyrosine kinases including PDGFR and c-Kit, in addition to VEGF signalling, it is difficult to interpret the mechanism of blood vessel development in the thymus.

4.4 Discussion

4.4.1 Blood vessel development in the thymus

Blood vessel development generally occurs by one, or a combination, of two mechanisms. Endothelial cells may sprout from existing vessels into immature structures that in turn attract perivascular cells to wrap around the newly formed structures, providing structural support. Alternatively, in the absence of existing vessels, endothelial precursors, or angioblasts, adhere to each other and assemble into new vascular structures. Blood vessel development in the thymus is thought to occur at around E14 in gestation by sprouting of existing vessels nearby to the capsule into the parenchyma. VEGF, produced by TECs and mesenchymal cells within the rudiment, is considered to be the main angiogenic stimulus for sprout migration into the organ. Very few reports have been published regarding thymic vascularisation during organogenesis, and the principle that vessel formation occurs by sprouting of vessels outside the thymus has not previously been questioned (42, 237).

Since it was found that NC derived cells differentiate into perivascular cells before the proposed time of vessel formation, it is possible that they may play a role in attracting endothelial sprouts into the rudiment and their

organisation. For this reason, the expression of a marker for blood vessel structures, endomucin, was examined. Similar to previously published reports, mature blood vessels were not detected in the thymus until E15.5. However, in contrast to the proposed mechanism where endothelial cells sprout into the organ, endothelial precursor cells were already present by E13.5 scattered throughout the rudiment. Thus, an immature vessel network may form *de novo* from precursor cells, which connect up with endothelial sprouts as they enter the rudiment. In support of this hypothesis were experiments with cultured foetal thymic lobes. Since tissues surrounding foetal lobes were removed before they were cultured, vascularisation could not occur from branching of existing vessels nearby the thymus. After four days in culture the control rudiments, which were not incubated with vascular inhibitor, had developed blood vessel structures which were endomucin^{+}. These vessels may have formed from endothelial precursor cells that were already present within the rudiment before it was dissected from the embryo. The origin of endothelial precursor cells is currently unknown, but a lineage analysis could be performed using an endothelial cell specific-Cre, such as Tie2-Cre (238), to activate the Rosa26 eYFP reporter. This method would allow tracing of the origin of blood vessel endothelial cells and the timing of their contribution to the thymic rudiment.

NC derived cells were already present within the thymic rudiment at E13.5, concurrent with endothelial precursor cells, forming a network throughout the

organ reminiscent of capillaries and expressing the pericyte marker, desmin. By examining markers for blood vessel endothelium and perivascular cells at different stages of thymus organogenesis, it was not possible to determine whether the endothelial vessel structures or NC derived perivascular cell network develops first. Thus, a number of possible mechanisms for thymic blood vessel development are possible. Endothelial precursor cells resident within the thymus may attract NCCs into the rudiment and, subsequently, induce their differentiation into perivascular cells. Equally, it is possible that NC derived cells, having already differentiated into perivascular cells, enter the rudiment first and influence the migration of endothelial cells and the development of vascular networks by the production of molecules including VEGF.

In order to determine which of these mechanisms is correct, a number of questions need to be addressed. For example, do blood vessels form in the thymus in the absence of NC derived perivascular cells? Or, conversely, do NCCs differentiate in the absence of endothelium? To address these two questions, thymic rudiments were examined in the absence of NCCs or by blocking blood vessel development *in vitro*. NCC-deficient embryos died at E12 and thus it was not possible to examine thymic development using this tool. However, it may be possible to use a Cre-inducible diphtheria toxin receptor (DTR) transgenic system to delete NCCs in FTOC, similar to the one described in Experimental Strategy 4.2C. In this system however, mice

express a transcriptional stop cassette flanked by two LoxP sites upstream of the Diphtheria Toxin Receptor (DTR), rather than the Diphtheria Toxin itself. Cre-mediated excision of the stop sequence, under the control of the Wnt1 promoter and regulatory elements, renders the cells sensitive to diphtheria toxin (DT), thus addition of soluble DT would selectively kill NC derived cells. Thymic rudiments could be taken at E13.5, before blood vessel development, and cultured in the presence of DT. After two to four days in culture, blood vessel development could be assessed by staining with antibodies and imaging by confocal microscopy.

To address the question as to whether NCCs differentiate and form their characteristic three dimensional networks of cells in the absence of blood vessels, vascular inhibitors were used. Development of blood vessels in FTOC was prevented using Sunitinib Malate. In these cultures, NCCs continued to differentiate into cells of perivascular character, expressing desmin, but they did not enter the rudiment and redistribute into a network. These results suggest that NCCs may have an intrinsic ability to form perivascular cells, or, that they had already differentiated before the rudiments were dissected from the embryos and cultured with vascular inhibitors. In contrast, NC derived cell migration into the rudiment did appear to require blood vessel formation. However, Sunitinib Malate may block other receptor tyrosine kinases in addition to those previously described. Thus,

these analyses were inconclusive in demonstrating a role of vascularisation in NCC colonisation of the thymus rudiment.

In order to determine the mechanism of blood vessel development in the thymus, a more detailed analysis of the kinetics of the emergence of endothelial and differentiation and colonisation of perivascular cells needs to be performed. Moreover, an earlier analysis of NCCs associated with the thymic rudiment at E11.5 and E12.5 may indicate when differentiation into perivascular cells occurs. In addition to inducing deletion of NC derived cells, it may be possible to ablate blood vessel endothelium using an endothelial cell-specific Cre mouse line crossed to the DTR expressing mice mentioned above. Thus, thymic rudiments could be cultured in the presence of DT, which would induce Cre-expressing endothelial cells to die. This would allow one to determine whether NC derived cell differentiation and colonisation of the thymus require blood vessel development.

4.4.2 PDGFRα/β expression on NCCs

Mesenchymal cells, which derive from the NC, surround the epithelium of the foetal thymus and express PDGFRα (226, 239), and play an important role in stimulating proliferation of the thymic epithelium (240). Jenkinson and colleagues showed that loss of PDGFRα^{+} mesenchyme is associated with a downturn in epithelial proliferation later in gestation and, thus, they

speculated that the main role of NC derived mesenchyme is to stimulate the proliferation of epithelial cells. Jenkinson and colleagues suggested that once epithelial cells are competent to support thymocyte maturation, NC derived mesenchyme is no longer required and is lost from the thymus. Similar to these results, NC derived mesenchymal cells in the embryonic thymus were found to express PDGFRα. In addition, they also expressed PDGFRβ at this stage. As mentioned in chapter three however, our experiments indicate that NC derived cells are not lost from the thymus and instead they differentiate into perivascular cells and continue to be present in the postnatal thymus at least to nine to ten months of age. The apparent contradiction between our data, which shows that NC derived cells remain in the post natal thymus and those already published that suggest that they are lost, may be explained by the downregulation of PDGFRα, whilst remaining PDGFRβ^{+}, in later stages of thymus organogenesis. Since PDGFRα was used in previous studies as a marker to detect NC derived cells, its absence in later embryonic and adult thymus was taken as evidence to support the hypothesis that NC derived cells were no longer required and were lost or replaced. However, it is shown here that NC derived cells lose expression of PDGFRα and thus is not a suitable marker to detect NC derived cells.

A similar downregulation of PDGFRα has also been observed during pericyte differentiation in the brain, indicating similarities in differentiation of perivascular cells in these two organs (241, 242). The reason for the change

in PDGF receptor expression in the brain is not clear, however *in vitro* experiments suggest that it could be due to a change in function of these cells. For example, PDGFs have been demonstrated to stimulate proliferation and inhibit differentiation of 3T3-Li and smooth muscle cells *in vitro* (243, 244). It is also a potent mitogen for myogenic C2C12 cells, but inhibits their differentiation into myotubes (245). Thus, the balance of expression between the two PDGF receptors may govern a cell's decision to proliferate or undergo growth arrest and terminal differentiation. For instance, in the thymus, loss of PDGFRα expression on NC derived mesenchymal cells correlates with their differentiation into perivascular cells. In order to test this hypothesis it will be necessary to determine the functional capacity of PDGFRα and –β expressing cells. This could be done *in vitro* by assessing the proliferation of thymic epithelial cells when cultured with α or β receptor expressing NC derived cells. Additionally, analysis of the expression profile of certain genes may indicate that those involved in vessel development or function are upregulated, whilst those involved in stimulating epithelial cell proliferation are downregulated in β receptor^{+} cells.

4.4.3 PDGFRβ$^{-/-}$ mice do not exhibit thymus defects

In mice deficient for PDGFRα expression on NCCs, a small number of thymi are hypoplastic due to a lack in epithelial cell proliferation, but are functionally competent (227). In contrast, broad deletion of PDGFRα results

in a significantly more severe phenotype of craniofacial and cardiac defects, including a lack of, or hypoplastic, thymic rudiments (226). The results from these experiments may seem to contradict each other, but can be explained by the different mutations in the two experimental systems. Broad deletion of PDGFRα is a result of the naturally occurring *Ph* mutation, which is a large deletion encompassing more than just the *PDGFRα* gene. It is also possible that PDGFRα was deleted in additional cell lineages, such as somite-derived mesoderm, which could have contributed to the severe craniofacial phenotype in mice with the broad deletion. Conversely, conditional deletion of the α receptor specifically in NCCs may have been incomplete with the remaining PDGFRα^{+} NCCs moderating the effects. These concerns make it difficult to ascertain that the phenotypes are due to the *PDGFRα* gene alone.

The function of PDGFRβ in thymus development has not previously been characterised. One of the ligands for this receptor, PDGF-B, is produced by endothelial cells resulting in the recruitment of pericytes to the walls of blood vessels. Elsewhere in the embryo, the absence of either PDGFRβ or PDGF-B is associated with a reduction in the number of perivascular cells associated with vessels and there is a defect in endothelial cell proliferation. Here it is shown that in the thymus of PDGFRβ$^{-/-}$ embryos, thymic blood vascular development and perivascular cell recruitment to vessels appears normal. These results appear to contradict evidence for the role of PDGF-B-PDGFRβ signalling in blood vessel development, but can be explained by

the redundant expression of PDGFRα on the perivascular cells, which also bind the PDGF-B ligand.

Redundancy between PDGF receptors was demonstrated in experiments where deletion of PDGFRβ resulted in a phenotype only in certain tissues (204). These authors suggest that if the downstream signalling pathways of α and β receptors are the same, the expression of PDGFRα should be able to rescue the mutant phenotype. They show that in the tissues where deletion of PDGFRβ does not affect blood vessel development, PDGFRα is also expressed, which assuages the vascular defects. Here, it is shown that the thymic blood vessels are likewise not affected by deletion of the β receptor. As NC derived mesenchymal cells are both PDGFRα$^+$ and β$^+$ at E15.5, when blood vessel development occurs, in the absence of PDGFRβ, PDGFRα may substitute its function and permit normal blood vessel development. Since the α receptor is subsequently down-regulated, it is possible that vascular defects in the thymus would present in the adult. Since β receptor mutants are neonatal lethal, these analyses were not possible. However, it may be possible to indirectly test this hypothesis by examining thymic rudiments from PDGF-B ligand deficient mice. If the signalling pathways downstream of the α and β receptors are the same, thus accounting for their redundancy, deletion of the B ligand will result in thymic vascular defects.

4.4.4 Summary

Previous results from chapter three indicated that NCCs differentiated into perivascular cells and were present in the thymic rudiment before the proposed immigration of endothelial sprouts. This result suggested that NC derived cells may play a role in blood vessel development and/or function. Vascularisation of the thymus was examined and endothelial precursor cells were found to be present inside the parenchyma at E13.5, suggesting that vessel development may occur by vasculogenesis, creating *de novo* structures that connect up with endothelial sprouts as they migrate through the capsule of the organ. It was not possible to determine the role of NC derived cells in this process, but their location and postulated expression of molecules required for vessel formation and function is intriguing and warrants further investigation.

Figure 4.1: PDGFRα and β are expressed in the perivascular compartment of the thymus. Thymi from three month old C57BL/6 mice were digested with collagenase, stained with antibodies recognising PDGFRα, β, NG2, CD31, CD45 and MHC II and analysed by flow cytometry. Cells were first gated PDGFRα$^+$ (blue line) or β$^+$ (red line) and further analysed for the expression of NG2, CD31, CD45 and MHC II.

Genotype of Mouse	% NG2	% CD31	% CD45	% MHCII
C57BL/6	73.9 52.6	11.7 10	9.05 15.5	6.6 18.7
C57BL/6	77.8 45.9	9.8 9.7	5.46 6.8	7.9 12.3
C57BL/6	72.1 53.8	12.1 13.5	8.2 12.9	4.5 9.1
C57BL/6	68.9 60.2	6.4 9.2	4.2 8.4	6.5 12.3

Figure 4.2: PDGFRα and β expression in embryonic and adult thymus. A Thymi from E15.5 (top) and adult (bottom) *Wnt1-Cre;Rosa26*^{eYfp} mice were digested with collagenase, stained for PDGFRα (left) and β (right) and analysed by flow cytometry. Isotype control, Goat IgG, is shown below. **B** Thymi from three month old adult *Wnt1-Cre;Rosa26*^{eYfp} mice were stained with antibodies detecting PDGFRα (left) and β (right) and analysed by flow cytometry. White arrows; capsule.

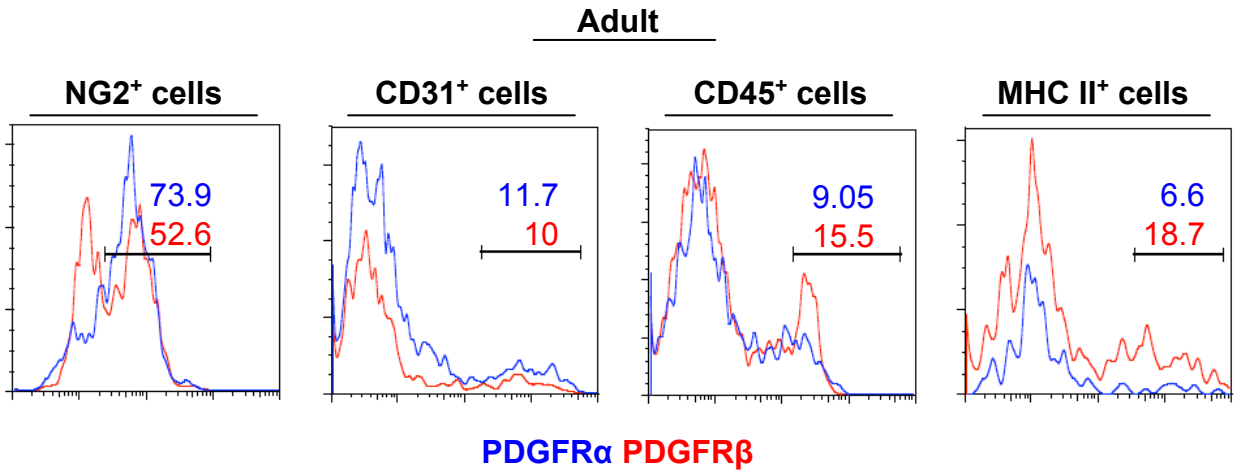
Genotype	E15.5 % PDGFRa	E15.5 % PDGFRb	Adult % PDGFRa	Adult % PDGFRb
Wnt1-Cre;Rosa26^{eYfp}	63.1	92.8	9.21	97.7
Wnt1-Cre;Rosa26^{eYfp}	89	95.2	12.8	98.1
Wnt1-Cre;Rosa26^{eYfp}	63.2	91.7	13.5	96.1

Figure 4.3: Normal thymus blood vessel development in PDGFRβ$^{-/-}$ mice. Thymi from E15.5 (top) and E18.5 (bottom) PDGFRβ$^{-/-}$ (left) and C57BL/6 (right) embryos were stained with antibodies recognising desmin (blue) and endomucin (red) and analysed by confocal microscopy. White arrows; capsule.

Figure 4.4: Endothelial precursor cells are present within the thymus by E13.5. Thymi from E13.5 (left) and E15.5 (right) C57BL/6 embryos were snap frozen in OCT embedding compound, sectioned and stained with antibodies detecting CD31 (red) and pan-cytokeratin (PanK, green) and analysed by confocal microscopy. Red arrows; endothelial cells. Green arrows; epithelial cells. This experiment was performed in collaboration with Julie Sheridan, University of Edinburgh.

Figure 4.5: Monoclonal antibody DC101 does not block blood vessel formation. Foetal thymic organ cultures were incubated with anti-human CD2 antibody (left) or DC101 antibody (right) for four days, stained with antibodies recognising eYFP (green) and endomucin (red) and analysed by confocal microscopy. Area within white box of upper panels are magnified in the lower panels. Results are indicative of three independent experiments consisting of two to three rudiments for each condition.

Figure 4.6: Blocking the formation of the blood vasculature with a biochemical inhibitor disrupts the NC derived cell network. (A) Foetal thymic organ cultures were incubated without inhibitor (left) or with Sunitinib Malate (right) for four days, stained with antibodies recognising eYFP (green) and endomucin (red) and analysed by confocal microscopy. Area within white box is magnified in the lower panels. **(B)** Foetal thymic organs cultured with (top two rows) and without (bottom two rows) Sunitinib Malate for four days, fixed and stained with antibodies recognising eYFP (left, green) and Desmin (middle, red). Merge of eYFP and Desmin (left). Results are indicative of four independent experiments consisting of three rudiments for each condition. Area within white box of upper panels is magnified in the lower panels.


Adult
NG2+ cells
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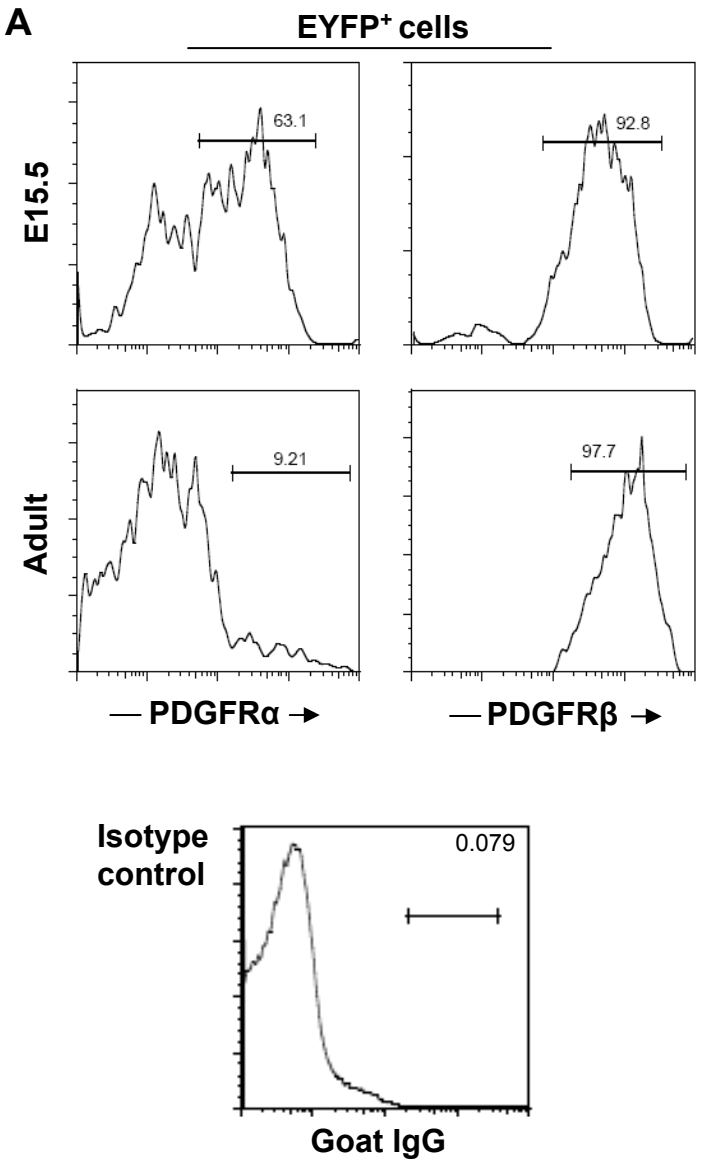
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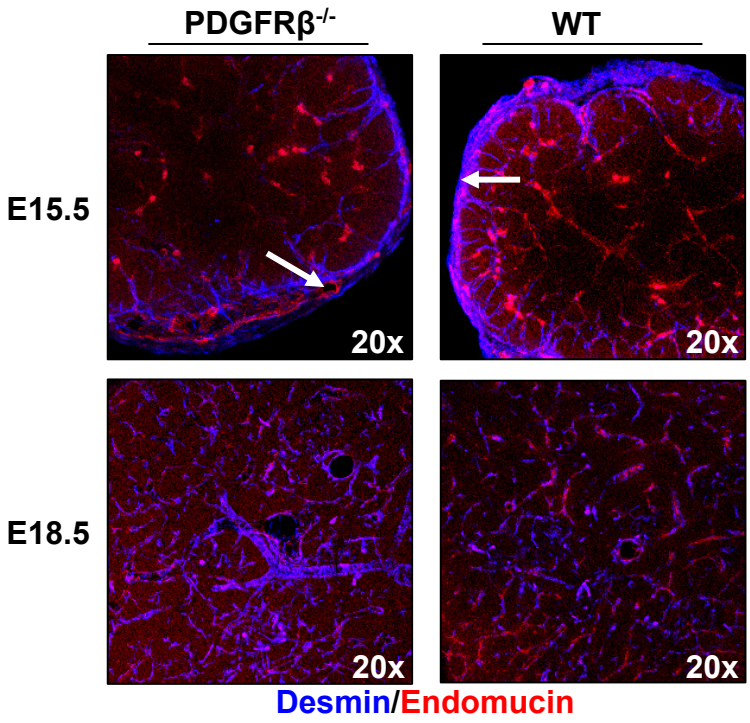
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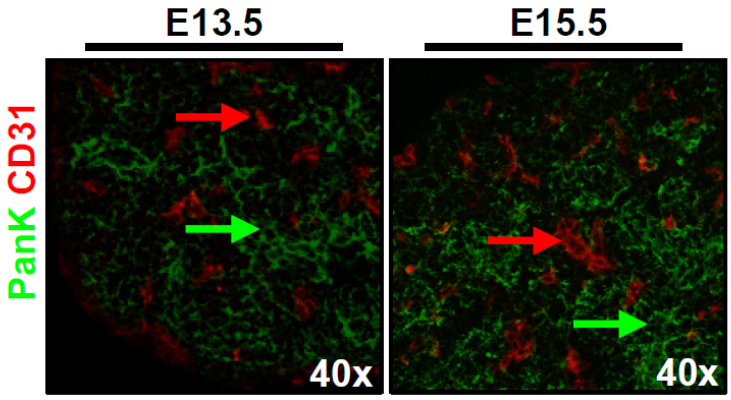
E13.5
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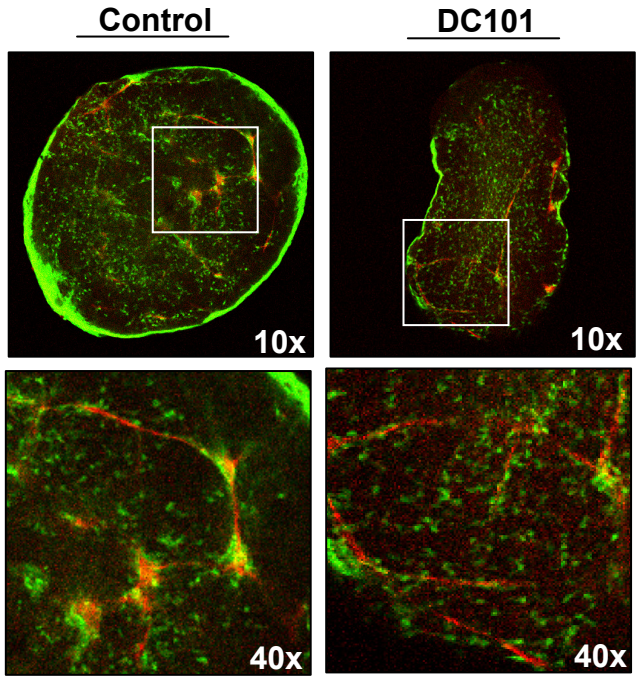
Control
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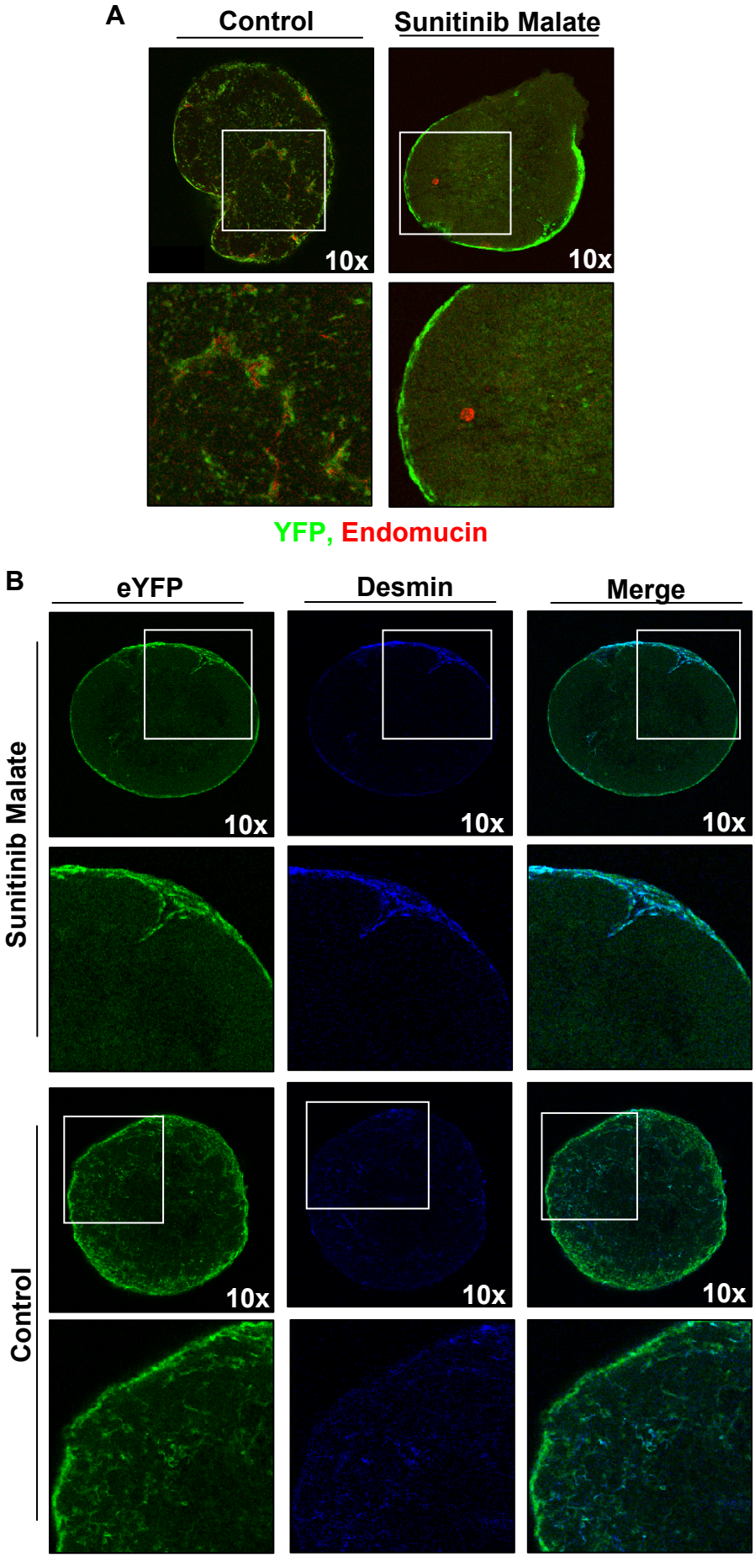
40x
YFP, Endomucin


A
Control
Sunitinib Malate
10x
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Chapter Five: Eph/ephrins are required for normal migration of the thymic rudiment.

In addition to the previously described roles of NC derived cells in the thymus, evidence is presented here indicating that they are further required for the relocation of the thymic rudiment to the thoracic cavity during organogenesis. NCCs express members of the Eph/ephrin family of signalling molecules, and in their absence the location of the thymus is abnormal. However, this is not due to a lack in NCC contribution to the organ. Thus, the ectopic location of the thymus in mice lacking ephrin-B2 expression on NCCs may result from impaired separation of the rudiment from the third pharyngeal pouch or impaired migration through the cervical structures into the thoracic cavity.

5.1 Experimental Rationale

Thymus development in the mouse begins at E9.5 with the formation of the third pharyngeal pouches and subsequent expression of transcription factors including Hoxa3, Pax1/9, Eya1 and Six1/4 within the third pharyngeal pouch endoderm, initiating the processes of patterning and initial organogenesis (3-8). Soon after, NC derived mesenchymal cells from the posterior hindbrain undergo EMT, delaminate, migrate towards and surround the pouch, which simultaneously begins to grow and bud into a separate structure containing the thymus and parathyroid primordia. By E12.5 the thymic primordium is completely detached from both the pharyngeal pouch and the parathyroid primordium. After separation, the bilateral lobes gradually descend possibly following the common carotid artery into the thoracic cavity where they finally settle in the upper mediastinum just above the heart at E13.5 (79) (Figure 5.1).

The function of NC derived cells in the patterning of the rudiment and its separation from the pharynx and/or parathyroid are unknown. Expression of the Eph family of tyrosine kinase receptors and their ligands, ephrins, may be attractive candidates since these molecules are required for a number of developmental processes, particularly patterning and morphogenesis. For example Eph/ephrins are implicated in; blood vessel formation through capillary sprouting (176); demarcation of arteries and veins (175-177) and

NCC migration to the pharyngeal arches (174, 180, 181, 184). The mechanisms by which Eph/ephrins control these processes are thought to involve regulation of actin cytoskeleton dynamics, cell-substrate adhesion, intercellular junctions, cell shape and cell movement (reviewed in (130, 131). Thus, Eph/ephrin expression by NCCs may have at least three roles in thymus development; NCC migration to the pharyngeal pouches where thymus development initiates, the separation of the combined thymus/parathyroid rudiment from the pharynx and the separation of thymus- and parathyroid-fated cells at the border between the two rudiments.

5.2 Experimental Strategy

Due to the importance of ephrin ligands in craniofacial development by influencing NCC migration and their role in thymus function by influencing the stromal compartment, their role in thymus organogenesis was investigated. A significant number of Eph receptors and ephrin ligands are expressed in the thymus (132-136), and are also known to have immuno-regulatory properties (140). The defects seen in thymocyte maturation in Eph or ephrin-deficient thymi are thought to result from abnormal development of the stromal cell compartments (196) and by modulation of T cell responses (246). For example; EphB2 and/or EphB3 deficient mice exhibit decreased numbers of thymocyte subsets (191); lack of EphA4 expression results in hypoplastic thymi and decreased numbers of double-positive ($CD4^+$ $CD8^+$)

thymocytes (196); and blocking foetal thymic organ cultures with EphB2/Fc or ephrin-B1/Fc fusion proteins decreases double positive and single positive T cell populations (190).

In order to determine whether ephrin-B2 and its preferred receptor EphB4 are expressed on TEC and NC derived cell thymic stromal populations, thymi from adult mice were digested with collagenase, stained with antibodies recognising CD45, ephrin-B2 and EphB4 and analysed by flow cytometry. To mark NC derived cells, expression of the yellow fluorescent protein (eYFP) reporter was activated with Cre recombinase expressed under the control of the Wnt1 promoter (198, 201). Previously published reports have shown this tool identifies reliably NC derived cells (213, 247). Cells were first gated on eYFP and CD45 expression and were further analysed for the presence of ephrin-B2 and EphB4 using antibodies recognising these molecules. In one such analysis, 84.3% and 94.8% of TECs ($CD45^-$ and $eYFP^-$) and 98.7% and 99.5% NC derived cells ($CD45^-$ and $eYFP^+$) expressed ephrin-B2 and EphB4 respectively (Figure 5.2A, B).

Since ephrin-B2 is highly expressed on the TEC and NCC compartments within the thymus, and NCC specific ephrin-B1 deletion has been described elsewhere (184), ephrin-B2 was specifically deleted from TECs and NCCs using Cre-Lox genetic tools. To this end, mice with LoxP sites flanking exon 2 of the *Efnb2* gene (Figure 5.3) (202) were crossed with mice expressing

Cre recombinase in TECs under the control of either the *IL7* (200) or in NCCs under the control of *Wnt1* (198) promoter and regulatory elements. Cre activity was reported via activation of a 'silent' enhanced yellow fluorescent protein (eYFP), expressed from the Rosa26 locus (201).

5.3 Results

5.3.1 ephrin-B2 expression on TECs is not necessary for correct positioning of the thymus and thymocyte development.

In order to study the role of ephrin-B2 on TECs, mice with LoxP sites flanking exon 2 of the gene were used (Figure 5.3A) (202). These were bred to mice in which Cre recombinase is expressed under the control of the *IL7* promoter (Figure 5.3B) (200). The *Rosa26*^{eYfp} reporter was also included in order to indelibly mark TECs and their progeny with eYFP. Deletion of ephrin-B2 from IL7^{+} TECs resulted in no gross abnormalities of the thymus. The organ was found in its normal anatomical location, was of normal size (Figure 5.4A) and the mice exhibited no apparent immunological defect. Thymi from mutant mice contained similar numbers of cells and proportions of the different sub-populations to those from control mice (Figure 5.4B) indicating no defect in thymocyte development. Moreover, peripheral T and B cell populations were very similar in cells obtained from the spleen of mice deficient in ephrin-B2 expression on TECs and wild type controls (Figure

5.5). Thus, ephrin-B2 expression on TECs is not required for thymus organogenesis, morphogenesis or TEC differentiation.

5.3.2 ephrin-B2 expression on NC derived cells is required for correct positioning of the thymus, but not for thymocyte development.

To study the role of ephrin-B2 expression on NC derived cells a similar strategy to that described in the previous section was used, except that Cre recombinase was expressed under the control of the *Wnt1* promoter. In mice that had deleted ephrin-B2 specifically from NC derived cells, the thymus was absent from its normal anatomical location above the heart (Figure 5.6A, bottom right). Instead, two large bilateral lymphoid structures were found in the cervical region (Figure 5.6A, top right). In a small number of embryos, one thymus lobe was found within the superior mediastinum above the heart, with a concomitant presence of a large lymphoid structure in the contralateral region of the neck.

In order to determine the cellular composition of these lymphoid structures they were dissected and digested with collagenase. The resulting single cell suspension was counted and stained with antibodies detecting CD4, CD8 (Figure 5.6B, top) and TCRβ (Figure 5.6B, bottom) to label T cells, and CD19 (Figure 5.7) for B cells, and analysed by flow cytometry. Expression patterns of T cell markers and a lack of B cell markers indicated that the

ectopic structures were thymus lobes and not lymph nodes. Moreover, normal cervical lymph nodes were found correctly located in mice with ectopic thymi. Peripheral T and B cell populations were also very similar in cells isolated from the spleen of mice deficient in ephrin-B2 expression on NC derived cells and wild type controls (Figure 5.8).

Thus, whereas absence of ephrin-B2 from NCCs does not affect the development of thymocytes, it results in ectopic thymus located in the cervical region.

5.3.4 NCCs colonise and differentiate normally within the thymic rudiment in the absence of ephrin-B2.

In *splotch* mice, which are Pax3 null, the observed ectopic location of the thymus is caused by a deficiency in NC derived mesenchymal cell migration to the third pouch. In order to determine whether ephrin-B2 deficient NCC mutants had a similar failure of NCC contribution, thymi from control and mutant three month old adult mice both expressing eYFP in NCCs were digested with collagenase and analysed by flow cytometry. One such analysis showed very similar numbers of $eYFP^+$ cells in the adult mutant, 5.8% (Figure 5.9A, right), compared to 6.4% in the control (Figure 5.9A, left). Additionally, thymi from E15.5 control *Ephrin-B2*$^{+/+}$*;Wnt1-Cre;Rosa26*^{eYfp} (Figure 5.9B, left) and mutant *Ephrin-B2*$^{Lx/Lx}$*;Wnt1-Cre;Rosa26*^{eYfp} (Figure

5.9B, right) embryos were dissected, fixed, stained with an antibody detecting YFP and imaged by confocal analysis. These analyses revealed that similar numbers of NC derived cells migrate into the mutant compared to control rudiments, thus, there are no changes in the colonisation of the primordium by NC derived cells.

NC derived cells in the thymus normally differentiate into cells characteristic of pericytes and smooth muscle cells associated with blood vessel walls (213, 247). ephrin-B2 and its preferred receptor EphB4 are known to play a role in normal blood vasculature development, and broad deficiency in these molecules is embryonic lethal (183). In order to determine whether ephrin-B2 expression is required for NC derived mesenchyme attachment to the walls of blood vessels, 100µm vibratome sections were examined from three month old adult control *Ephrin-B2*$^{+/+}$*;Wnt1-Cre;Rosa26*^{eYfp} and mutant *Ephrin-B2*$^{Lx/Lx}$*;Wnt1-Cre;Rosa26*^{eYfp} thymi. The sections were stained with antibodies detecting eYFP, the pericyte marker desmin, and the endothelial marker endomucin. These analyses revealed that blood vessels in the mutant thymus develop normally with desmin^{+} perivascular cells surrounding endomucin^{+} vascular endothelium (Figure 5.10A, right) when compared to control thymi (Figure 5.10A, left). In addition, detection of eYFP^{+} perivascular cells in the adult thymus suggests that they are NC derived and are not replaced by cells of other mesenchymal origin with age (Figure 5.10B).

Wnt1 is expressed on neurons in addition to other NC derived tissues. Thus, the neurons that innervate the thymus may also be affected by the deletion of ephrin-B2. In order to determine whether the neuronal component of the thymus was compromised in the mutant mice described here with ectopic thymi, sections of thymus from three month old adult control *Ephrin-B2*$^{+/+}$*;Wnt1-Cre;Rosa26*^{eYfp} and mutant *Ephrin-B2*$^{Lx/Lx}$*;Wnt1-Cre;Rosa26*^{eYfp} mice were stained with antibodies detecting TUJ1, a neuronal marker, and eYFP. Similar patterns of TUJ1 staining were observed in the mutant mice when compared with control thymi, thus, it appears that ephrin-B2 is not required for innervation of the thymus (Figure 5.11).

Together these analyses revealed that ephrin-B2 deficient NC cells delaminate normally, migrate to the thymic rudiment and form a three dimensional network of cells, similar to that observed in control embryos and reported previously (213, 247). Thus ephrin-B2 appears not to be necessary for NCC association with the rudiments and this raises the possibility that other ephrin molecules are involved in this process.

5.4 Discussion

5.4.1 The ectopic location of the thymus

There are a number of key steps that occur during thymus organogenesis, starting with the initial induction of a common primordium containing the thymus- and parathyroid-specific domains from the endoderm of the third pharyngeal pouch. Next, patterning of the common primordia into organ-specific domains from morphologically indistinguishable epithelial cells is established. The shared primordia then detach from the pharyngeal endoderm at around E12, followed by separation of the thymus and parathyroid rudiments from each other. Once separated, the thymus migrates down into the superior mediastinum where it finally rests above the heart. Shortly after thymus migration is complete, the thoracic cavity closes by midline fusion of paired cartilage bars (79). The mechanism leading to the collective migration of the thymus primordium is unknown. Several different mouse mutants have been described that have ectopic thymi located in the neck. These mutants have complex defects involving predominantly patterning and separation of the rudiment from the pharynx and/or the parathyroid (See table for details and references).

For example, Hoxa3 mutant mice exhibit a very early failure of thymus organogenesis, in fact, initiation of the rudiment at the third pouch does not occur (3). Deletion of one allele of Hoxa3 in Hoxb3$^{-/-}$ Hoxd3$^{-/-}$ mutant mice, however, resulted in the formation of an ectopic thymic rudiment in the cervical region (22). Additionally, RARα/γ double mutants also have ectopic organs, and it is thought that this is due to the lack of activation of *Hox* genes

by retinoic acid (248). Likewise, Pbx1 mutant mice also exhibit early defects in thymus formation. In these mice, there is delayed or absent formation of the pharyngeal pouches and, in particular, disorganised patterning of the third pouch (249). In a few mutants, a thymus does arise, but it is often ectopic and the thymic epithelium does not expand. Due to the similarities between the Pbx1 and Hoxa3$^{+/-}$ Hoxb3$^{-/-}$ Hoxd3$^{-/-}$ compound mutants, it was suggested that these genes act together in the development of the pharyngeal pouches. Thus, defects in the initial formation of the rudiment are implicated in some of the mutants with ectopic thymi.

Defects that result in ectopic thymi can also manifest later in organogenesis, as a result of the aberrant separation of the rudiment from the pharyngeal pouch and/or the parathyroid. Hox3$^{+/-}$ Pax1$^{-/-}$ mutants, in addition to impaired development of appropriate numbers of epithelial cells causing hypoplasia, exhibit a delay in the separation of the combined thymus and parathyroid rudiment from the pharynx (5). By the time separation has occurred, the environment though which the thymic rudiment migrates has developed further, preventing movement of the anlagen as a secondary defect. Likewise, in Pax9 null mice, the thymus develops as an ectopic polyp-like structure in the larynx as a result of impaired separation of the rudiment from the pharynx. Whilst the rudiment is colonised by mesenchymal and lymphoid cells, it is also severely reduced in size from E14.5 onwards (21). The ectopic location of the thymus in *Splotch*, or Pax3 null, mice is thought to

result from abnormal boundary formation between the thymus- and parathyroid-domains and thus a defect in the separation of the two organs, which holds the thymus in the neck. Interestingly, this defect was attributed to a deficiency in NCCs that surround the rudiment and contribute to boundary formation between the two domains. Alk5 mutants, which have a mutation in the TGFβ-type1 receptor on NCCs, also exhibit ectopic thymi as a result of delayed separation of the parathyroid and thymus. In these mutants the NCCs migrate normally to the pouch, but it is thought that an increase in apoptosis of postmigratory NCCs perturbs separation and as a result the thymic rudiment is held in the cervical region (25). FGFs have also been implicated in the separation of the rudiment from the pharynx, since Sprouty mutants, which are deficient in FGF signal transduction, also exhibit ectopic thymi that remain attached to the pharynx (250).

Ectopia of the thymus can thus result from defects in early stages of thymus/parathyroid patterning or impaired separation of the combined rudiment from the pharynx and from each other. In addition to separation from the pharynx, the thymus rudiment must undergo a process of migration into the thoracic cavity, however, the mechanism of migration is unknown and, to date, no mutants have been described which have a primary defect in rudiment migration without major earlier defects in rudiment formation and patterning.

Interestingly, a number of the mutants described with ectopic thymi are thought to result from defects in functions of the NC derived cell population. Likewise, the mutants described here lack expression of ephrin-B2 on NCCs and have ectopic thymi in the cervical region. In contrast to some of the mutants described above, the thymus in the mutants described in this study appears to function normally and is not reduced in size compared to wild type control organs. To our knowledge, this is the first report demonstrating a role for the Eph/ephrin family of molecules and NC derived cells in the positioning of the thymus. Since Eph/ephrins are required for NCC migration, the abnormal location of the thymi may result from a lack of NC derived cell contribution to the rudiment. However, mutant NCCs were found to associate with the ectopic thymus in similar numbers to wild type NCCs and formed three dimensional networks normally. Thus, the position of the thymus may result from either a delay or defect in the separation of the rudiment from the pharynx and/or the parathyroid, or an impairment in organ migration. This question, as to whether impaired separation or migration causes the ectopic location in the absence of ephrin-B2 expression on NCCs, will be explored in the next chapter.

5.4.2 NC derived cell function in the ectopic thymus

Eph/ephrins are known to play a key role in the migration of NCCs, dictating the timing and patterning of their migration. Knocking out ephrin-B1 on NCCs

resulted in defects in pharyngeal organ development including a cleft palate. Likewise, these defects were caused by abnormal migration of the NCCs, thus, instead of migrating along their specified pathways they seemed to exhibit a wandering behaviour and invaded improper territories normally devoid of NCCs (184). In the mouse, ephrin-B2 is expressed on the underlying mesoderm along the pathway which NCCs migrate (174). Thus, deletion of the ligand prevented NCC migration into the second pharyngeal pouch but NCCs migrated normally to the third pharyngeal pouch. ephrin-B2 has not been deleted on NCCs specifically, but mice deficient for ephrin-B2 on all cells have been described (176).

For this reason ephrin-B2 was deleted specifically from NCCs, using a similar strategy to that used previously to delete ephrin-B1 on these cells. Thus, mice with exon two of the ephrin-B2 gene floxed with LoxP sites on both sides, were crossed with mice expressing Cre recombinase under the control of the Wnt1 promoter and regulatory elements. In contrast to ephrin-B1 deficient NCCs, ephrin-B2 deficient NCCs still appeared to migrate to third pouch. Thus, the ectopic thymic rudiment was surrounded and invaded by NCCs in a similar pattern to that seen in control embryos and those described in chapter 3 of this thesis. Thus, NCC migration was not affected in the absence of ephrin-B2 indicating that other Eph/ephrin molecules were probably required for this function. It will be interesting to see the effect of

deletion both ephrin-B1 and ephrin-B2 on NC derived cells and TECs, thus these mice are currently being generated.

What perhaps is surprising from these results is that blood vessels in the thymus of mice that were deficient for ephrin-B2 expression on NCCs did not appear abnormal. In mice deficient for ephrin-B2 or its preferred receptor, EphB4, on all cells, angiogenic remodelling is defective. Moreover, when ephrin-B2 was specifically deleted on perivascular cells using a similar strategy to that used here, blood vessels frequently ruptured releasing blood cells into the surrounding tissue. In contrast, it is shown here that deletion of ephrin-B2 on NC derived perivascular cells does not cause an obvious defect in vessel structure and function. The differences between these two results may be due to the deletion of Cre on different subsets of perivascular cells. Previous experiments deleted ephrin-B2 using PDGFRβ-Cre, whilst here an NC specific Wnt1-Cre was used to drive deletion of the ligand. It is possible that not all of the perivascular cells in the thymus are derived from the NC, therefore driving ephrin-B2 deletion with the PDGFRβ-Cre may affect more cells than deleting the ligand just on those deriving from the NC. It is possible that there are subtle defects in perivascular cell attachment to endothelial cell-vessel walls that become more apparent with age. Indeed, a number of adult mutant mice described here die at 8-12 weeks of age due to haemorrhaging in the brain, thus vessel defects do become apparent with age. To investigate this further, it will be necessary to compare vessel

structure by TEM analysis of aged mutant and control mice. Moreover, H&E analysis of thymus sections from aged mice may show haemorrhaging into the parenchyma.

5.4.3 T cell development in the absence of ephrin-B2

Most Eph receptors and ephrin ligands are known to be expressed in the thymus in an overlapping pattern (135, 251). Several Eph and ephrins can appear on the same thymic compartment and at the same developmental stage, allowing for different combinations of signals and there is functional redundancy between different receptors or ligands (138, 252). T cell development requires contact between stromal cells and thymocytes, and this cell contact-dependent mechanism appears to require the expression of a number of Eph/ephrins. For example, ephrin-B1 and its receptors are critical for T cell development (138). Thus, perturbing Eph/ephrin interactions in foetal thymic organ culture with fusion proteins interferes with thymocyte survival and maturation. EphB receptors modulate T cell responses in a number of ways; they cluster with activated T cell receptors in aggregated lipid rafts, and clustering with immobilised anti-EphB6 antibodies or ephrin-B Fc fusion proteins lowers the activation threshold of T cells responding to suboptimal TCR ligation (140, 190, 196). Moreover, EphB activation promotes T cell proliferation, production of interferon γ and cytotoxic T cell activity, and EphB6 knockout mice show impaired cellular immune

responses despite having normal T cell numbers (252). EphA4 knockout mice exhibit defects in thymocyte maturation and have greatly decreased numbers of peripheral T cells. Interestingly, these defects appear to result from abnormal development of the stromal cells of the thymus (196). Recently, it was reported that EphB2 and EphB3 double knockout mice also have disorganised thymic architecture and decreased numbers of thymocytes (253), thus Eph/ephrin signalling clearly plays some role in thymus organogenesis and function.

Although most Eph/ephrins have been described to be expressed within the thymus, it is unclear which cell type within the organ expresses ephrin-B2. Yu and colleagues (136) reported that ephrin-B2 mRNA was prominent in the thymic cortex, and was present at the protein level mainly on monocytes, macrophages and T cells (7.1%). Moreover, these authors also reported that the receptors for ephrin-B2 were expressed on T cells. In contrast, here it is shown that few (2.2%) T cells were positive for ephrin-B2 but, similar to previously published reports, more (14.4%) were EphB4^{+}. The differences between the number of T cells which express ephrin-B2 in the results reported here and those previously published may be due to the differences in experimental procedure. In the report by Yu and colleagues, ephrin-B2 expression was measured on T cells stimulated with anti-CD3 in culture. Thus, it will be necessary to isolate T cells from mice in which ephrin-B2 was

deleted on either NCCs or TECs and culture them in conditions described in the report by Yu and colleagues.

To our knowledge, this is the first report in which ephrin ligands have been deleted specifically from the stromal compartments of the thymus. Considering the large amount of evidence supporting a role for Eph/ephrin signalling in thymus development and function, it is perhaps surprising that deletion of ephrin-B2 from NC derived cells and TECs did not result in an overt immune phenotype. In these mice, the proportions of SP and DP T cells were not altered, and these mice did not exhibit any obvious immune defects. However, these mice were not immunologically challenged, and it is possible that subtle defects in TCR signalling or T cell proliferation or cytokine production may be present in these mice.

It is equally possible that functional redundancy between ephrin ligands compensated for the loss of ephrin-B2 in these mice, or that ephrin-B2 expressed by stromal cells does not have a role in thymus function. The PDZ-binding domains of all three ephrin-B ligands are 100% identical, indicating that they might activate indistinguishable signalling cascades. Additionally, ephrin-B1 and -B2 can bind to similar EphB receptors, although ephrin-B2 has a higher affinity for EphB4, and both genes are co-expressed in a number of tissues and cells during mouse embryonic development. Moreover, functional redundancy has been previously demonstrated for Eph

receptors and GPI-linked ephrins (the ephrin-As) (254, 255). However, ephrin-B1 and -B2 null mice exhibit different phenotypes and thus have specific, non-redundant functions (184, 185, 256, 257).

In order to clarify the role of specific Eph/ephrins in thymus development, it will be necessary to delete one or more Eph/ephrins on both the stromal and thymocyte compartments. For instance, deletion of both ephrin-B1 and –B2 on NCCs or TECs may result in defects in thymus development or function that was not apparent when deleting ephrin-B2 alone. Moreover, it will be necessary to challenge the immune system, and test TCR signalling, proliferative capacity and cytokine secretion of T cells from these mice.

Thymus function in an ectopic location

Similar to reports that the cervical thymus functions equally as well as the thoracic thymus (258, 259), our data indicates that the location of the organ bears little, if any, functional consequence. It has been demonstrated that cervical thymi from adult Balb/C mice grafted under the kidney capsule of adult C57BL/6 nude mice are capable of reconstituting the immune system. In contrast to transplanted foetal thymus grafts, cervical thymus grafts did not grow but, similar to foetal grafts, they were able to support thymocyte selection and export T cells bearing diverse TCRs into the peripheral lymphoid organs. Likewise, ectopic thymi, which resulted from the NC

specific deletion of ephrin-B2, apparently function normally and this raises the interesting question of; why does the thymus migrate during organogenesis?

The location of the organ is only one of a number of differences between thymi of different species. The origin of the gland from the pharyngeal pouches varies between species, as does the final number of organs. Thus, the thymus arises from pouches two to six in cartilaginous fish, from the 2nd in frogs, 2nd and 3rd in reptiles and 3rd or 4th in bony fish, birds and mammals. Sharks have five pairs of thymic glands, whilst caecilian amphibians have four pairs, urodele amphibians have three pairs and many teleost fish species, anuran amphibians and many mammals have just one pair. The position of the thymus also varies between species and it only migrates into the thoracic cavity of higher vertebrates. For instance, the koala has a cervical thymus, the kangaroo has both cervical and thoracic thymi, and cattle and sheep often have a thoracic and cervical organs, which may be connected (260).

One possible reason for the thoracic location of the thymus may be simply that there is not enough space in the cervical region. In support of this hypothesis are numerous reports of cervical thymus tissue causing respiratory distress in young children, as a result of narrowing or displacement of the airway. However, the mice with ectopic thymi that are

described here do not exhibit stridor, thus, it is likely that there are other reasons that account for the thoracic location of the thymus.

5.5.5 Summary

Deletion of the ligand ephrin-B2 from NC derived cells causes ectopia of the thymus. Confocal microscopy and flow cytometry indicated that this is not due to a lack of NCC contribution to the rudiment. Thus, the position of the thymus does not appear to impact on the function of the organ. Furthermore, it was shown that ephrin-B2 expression on NCCs is not required for T cell development. Thus, a role for Eph/ephrin signalling on NCCs in the separation from the pharynx and/or the parathyroid or migration of the rudiment into the thoracic cavity is likely.

Figure 5.1. Thymus formation and migration. A E11.5-12.5: Outgrowth and Patterning of the Rudiment. Specification of the rudiment into thymus- and parathyroid-specific domains. Patterning begins at E10 with the expression of GCM2 (red) in the third pouch, controlled at least in part by the Hox-Pax-Eya-Six cascade. High-level expression of Fonx1 (blue) begins at E11.25. **B E12.5 Separation from the Pharynx and Migration of the Rudiment.** Prior to migration, the rudiment must separate from both the pharynx and the parathyroid. (Adapted from Figure 5 of {Blackburn, 2004 #510}). **C E12.5-E13.5 Caudal and Ventral Migration.** Once separated from the pharynx and parathyroid, the thymic rudiment migrates down into the thoracic cavity, following the common carotid artery. **D E13.5 Final Resting Place Above the Heart.** The thymic rudiments reach their final destination, just above the heart and fuse in the midline by E13.5.

Figure 5.2. Expression of ephrin-B2 ligand and EphB4 receptor on NC derived mesenchyme and TECs. Thymus lobes from E15.5 *Wnt1-Cre;Rosa26*^{eYfp} embryos were digested with collagenase, stained with antibodies recognising CD45, ephrin-B2 and EphB4 and analysed by flow cytometry. Cells were first gated CD45^{+} (thymocytes) (A, B left), eYFP^{+} (NC derived) (A, B middle) or eYFP^{-}CD45^{-} (predominantly thymic epithelium) (A, B right) cells and further analysed for expression of ephrin-B2 **(A)** and EphB4 **(B)**. Red line; specific antibody, black line; isotype control.

Figure 5.3. Expression construct of ephrin-B2$^{Lx/Lx}$ and IL-7 Cre mice. A The expression construct used to generate mice with exon 2 of the *ephrin*-B2 gene floxed with LoxP sites. Top; untargeted locus, bottom; targeting locus. LoxP sites indicated by solid arrow-heads. Adapted from Grunwald *et al* 2004. **B** IL-7 expression construct generated in which Cre was inserted into the locus, replacing exon 1 of the gene. Top; untargeted locus, middle and bottom; targeted locus. Adapted from {Repass, 2009 #302}. Filled boxes indicate exons.

Figure 5.4: The thymus appears normal in the absence of ephrin-B2 expression on TECs. A Three month old adult *Ephrin-B2$^{+/+}$;IL7-cre* (left) and *Ephrin-B2$^{Lx/Lx}$;IL7-Cre* (right) mice were dissected to reveal the thoracic cavity in order to visualise the location of the thymus lobes. Red arrows; thymus, green arrows; heart. **B** Thymi from three month old *Ephrin-B2$^{Lx/+}$* and *Ephrin-B2$^{Lx/Lx}$;IL7-Cre* mice were dissected, digested with collagenase, stained with antibodies recognising CD4, CD8 (top) and TCRβ (bottom) and analysed by flow cytometry. Similar results were obtained in two independent experiments consisting of six and four mice respectively.

Mouse ID	CD4+CD8-	CD8+CD4-	CD4-Cd8-	CD4+CD8+	TCRb+
EphrinB2$^{+/+}$;IL7-Cre	7.3	4.2	3.4	85	18.9
	7.1	3.9	3.7	85.3	15.2
	8.1	4.1	3.9	83.8	17.5
	7.4	4	3.2	85.4	17.9
EphrinB2$^{Lx/Lx}$;IL7-Cre	11	5.2	3.5	81	13.6
	8.5	4.9	3.9	82.6	18.2
	7.4	3.9	3.2	85.5	15.1
	7.9	5.1	2.9	84	12.9
	10.2	5.6	4.9	79.3	17.5
	10.1	5.4	5	79.5	16.2

Figure 5.5: Normal peripheral T and B cell populations in the absence of ephrin-B2 expression on TECs. Spleens from three month old adult *Ephrin-B2$^{+/+}$;IL7-cre* (top), *Ephrin-B2$^{Lx/Lx}$;IL7-Cre* (middle) and C57Bl6 (bottom) mice were dissected, digested with collagenase and stained with antibodies recognising CD4 and CD8 (left column), TCRβ (middle column) and CD19 (right column). Similar results were obtained from three mice of each genotype.

Mouse ID	CD4+ CD8-	CD8+ CD4-	CD4- CD8-	CD4+ CD8+	TCRb+	CD19
EphrinB2$^{+/+}$; IL7-Cre	16.5	10	73.4	0.12	28.2	54.1
	17.8	15	67.2	0.1	29.5	56.3
	15.9	12.9	71.05	0.15	28.3	53.8
EphrinB2$^{Lx/Lx}$; IL7-Cre	16.8	16.7	66.3	0.12	35.4	53
	16.5	12.1	71.3	0.12	29.5	53.9
	17.2	16.8	66	0.1	32.9	53.6

Figure 5.6: Expression of ephrin-B2 on NC derived cells is required for the normal anatomical position of the thymus. A Three month old adult control *Ephrin-B2$^{+/+}$;Wnt1-Cre;Rosa26^{eYfp}* (left) and mutant *Ephrin-B2$^{Lx/Lx}$;Wnt1-Cre;Rosa26^{eYfp}* (right) mice were dissected to reveal the cervical region (top) and thoracic cavity (bottom) in order to visualise the location of the thymus lobes. White arrows; clavicle, red arrows; thymus, green arrows; heart. **B** Thymi from three month old control *Ephrin-B2$^{Lx/Lx}$* and mutant *Ephrin-B2$^{Lx/Lx}$;Wnt1-Cre;Rosa26^{eYfp}* mice were dissected, digested with collagenase, stained with antibodies recognising CD4, CD8 (top) and TCRβ (bottom), and analysed by flow cytometry. Similar results were obtained from more than three experiments consisting of fourteen *Ephrin-B2$^{Lx/Lx}$;Wnt1-Cre* mice.

Mouse ID	CD4+ CD8-	CD8+ CD4-	CD4- CD8-	CD4+ CD8+	TCRb+
EphrinB2$^{+/+}$	6.3	1.6	2.4	89	12.7
	7.2	3.4	3	86.4	15.8
	8.9	3.9	2.9	84.2	13
EphrinB2$^{Lx/Lx}$;Wnt1-Cre	5.9	1.6	2.3	90	17.9
	7.2	3.9	2.9	85.9	15.4
	6.1	3.1	3.1	87.7	13.9

Figure 5.7. Ectopic structures are not lymph nodes. Thymi from adult control *Ephrin-B2*$^{Lx/Lx}$ (left) and ectopic structures from mutant *Ephrin-B2*$^{Lx/Lx}$*;Wnt1Cre;Rosa26*^{eYfp} (right) mice were digested with collagenase and stained with an antibody detecting CD19 and analysed by flow cytometry. Isotype control (Mouse IgA) is shown. Similar results were obtained from two experiments consisting of six *Ephrin-B2*$^{Lx/Lx}$*;Wnt1Cre;Rosa26*^{eYfp} mice and three controls.

Mouse ID	% CD19
Ephrin-B2$^{Lx/Lx}$	0.1
	0.2
	0.05
Ephrin-B2$^{Lx/Lx}$;Wnt1Cre;Rosa26eYFP	0.1
	0.1
	0.05
	0.1
	0.2
	0.1

Figure 5.8: Normal peripheral T and B cell populations in the absence of ephrin-B2 expression on NC derived cells. Spleens from three month old adult *Ephrin-B2$^{+/+}$;Wnt1-cre* (top), *Ephrin-B2$^{Lx/Lx}$;Wnt1-Cre* (middle) and C57Bl6 (bottom) mice were dissected, digested with collagenase and stained with antibodies recognising CD4 and CD8 (left column), TCRβ (middle column) and CD19 (right column). Similar results were obtained from three mice of each genotype.

Mouse ID	CD4+ CD8-	CD8+ CD4-	CD4- CD8-	CD4+ CD8+	TCRb+	CD19
EphrinB2$^{+/+}$; Wnt1-Cre	19.4	8.5	71.9	0.15	27.8	62.6
	15.8	13.2	70.9	0.1	23.9	59.8
	19.1	12.3	68.6	0.15	27.3	60.5
EphrinB2$^{Lx/Lx}$;Wnt1-Cre	16.3	7.62	75.9	0.2	23.2	65.2
	14.9	12.9	72.1	0.15	26.9	60.5
	16.9	9.5	73.5	0.15	22.5	64.3

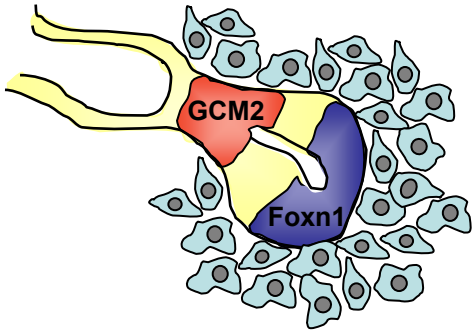
Figure 5.9: NC derived mesenchymal cells associate normally with the ectopic thymus. Thymi from three month old adult control *Ephrin-B2$^{Lx/+;}$Wnt1-Cre;Rosa26^{eYfp}* (**A, B** left) and mutant *Ephrin-B2$^{Lx/Lx}$;Wnt1-Cre;Rosa26^{eYfp}* mice (**A, B** right) were dissected, digested with collagenase and analysed by flow cytometry **A** or fixed with 4% paraformaldehyde, stained with an anti-eYFP antibody and analysed by confocal microscopy for the presence of eYFP+ cells. **B**.

Mouse ID	% YFP
Ephrin-B2$^{Lx/+}$;Wnt1Cre;Rosa26eYfp	6.4
	5.9
Ephrin-B2$^{Lx/Lx}$;Wnt1Cre;Rosa26eYfp	5.8
	5.7
	6.1

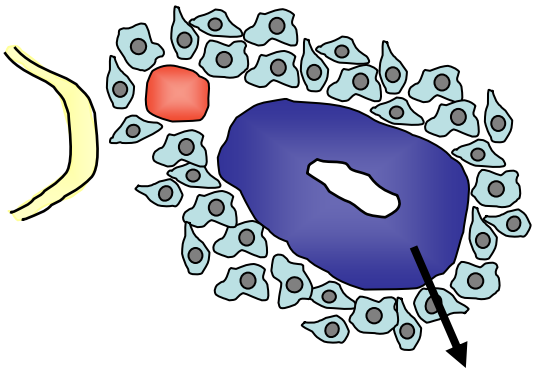
Figure 5.10: NC derived cells associated with the ectopic thymus differentiate normally into perivascular cells. A Thymi from three month old adult control *Ephrin-B2*$^{Lx/Lx}$ (left) and mutant *Ephrin-B2*$^{Lx/Lx}$*;Wnt1-Cre;Rosa26*^{eYfp} (right) mice were dissected, fixed in 4% paraformaldehyde, cut into 100µm sections, stained with antibodies recognising endomucin (red) and desmin (blue) and analysed by confocal microscopy. **B** Thymi from three month old adult control *Wnt1-Cre;Rosa26*^{eYfp} (left) and mutant *Ephrin-B2*$^{Lx/Lx}$*;Wnt1-Cre;Rosa26*^{eYfp} (right) mice were dissected, fixed in 4% paraformaldehyde, cut into 100µm sections, stained with antibodies recognising eYFP (green) and endomucin (red), and analysed by confocal microscopy.

Figure 5.11 Ablation of ephrin-B2 from NC derived cells does not affect the neurons of the thymus. Three month old adult thymus from control *Ephrin-B2$^{+/+}$;Wnt1-Cre;Rosa26^{eYfp}* (left) and mutant *Ephrin-B2$^{Lx/Lx}$;Wnt1-Cre;Rosa26^{eYfp}* (right) mice were fixed with 4% paraformaldehyde, cut into 100μm sections, stained with antibodies recognising Tuj1 (neurons, red) and eYFP (green), and analysed by confocal microscopy. Top panels; three-dimensional reconstruction of 100μm section. Lower panels; single confocal sections.

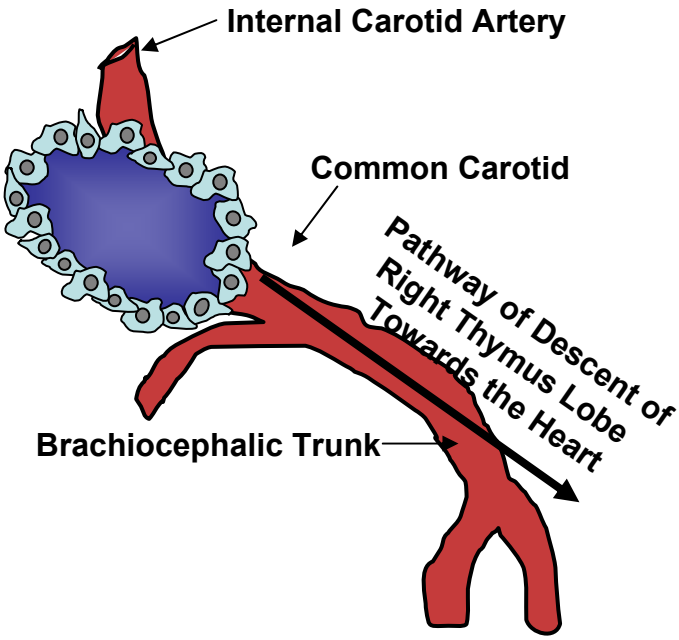
A E11.5-12.5: outgrowth and patterning



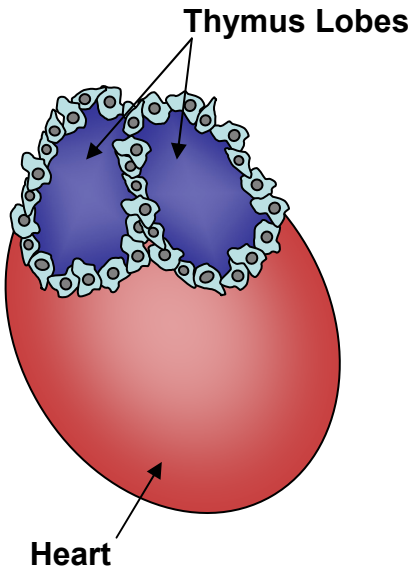
B E12.5: separation

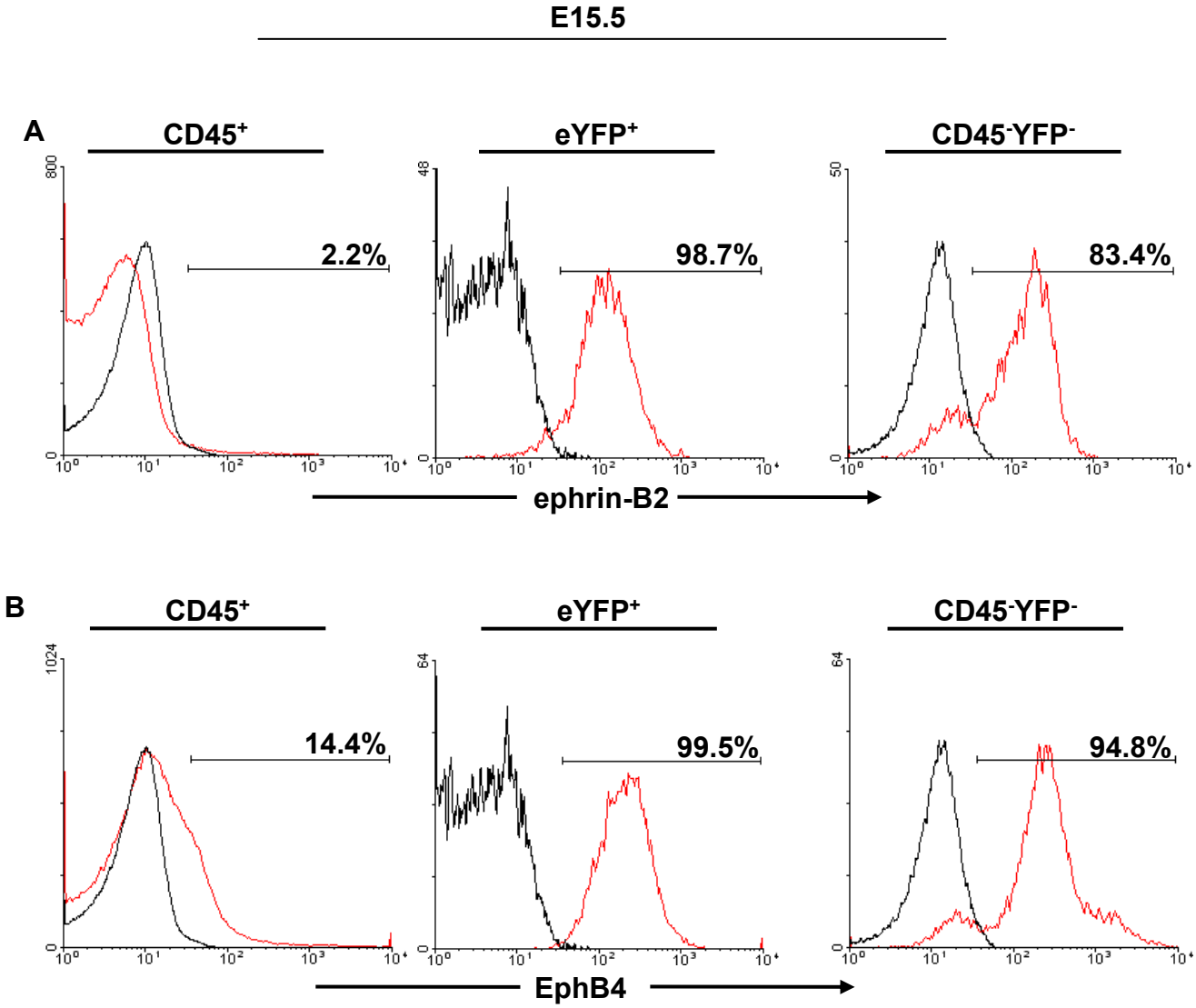


C E12.5-E13.5 Caudal and Ventral Migration

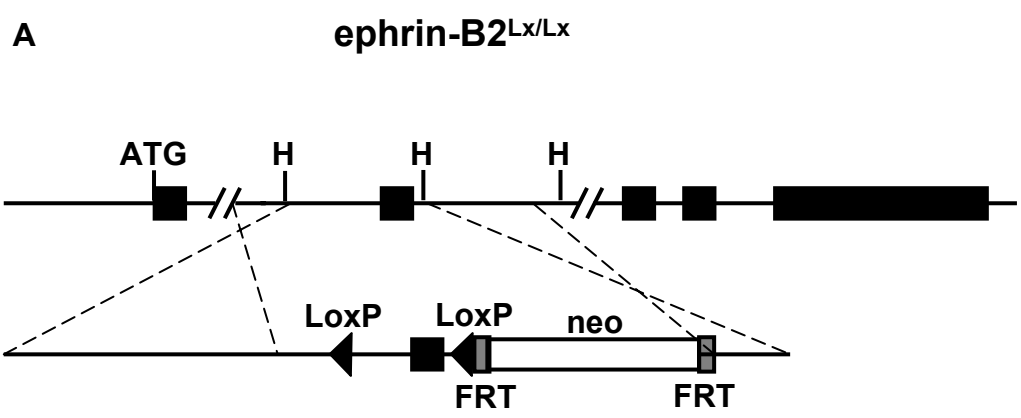


D E13.5 Final Resting Place Above the Heart

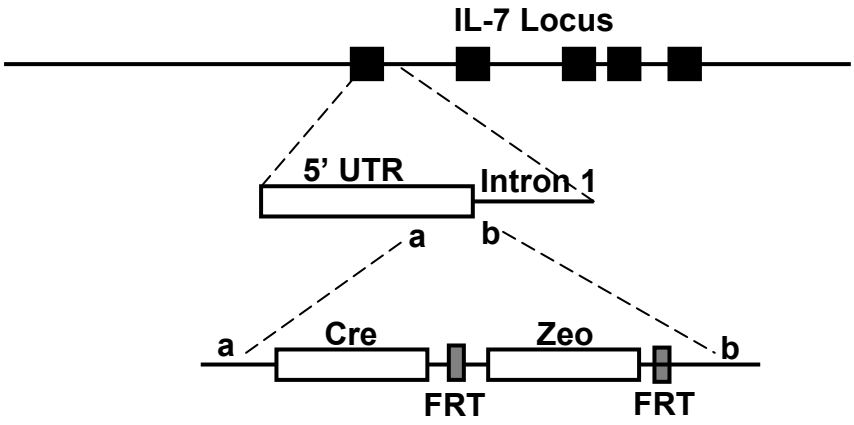


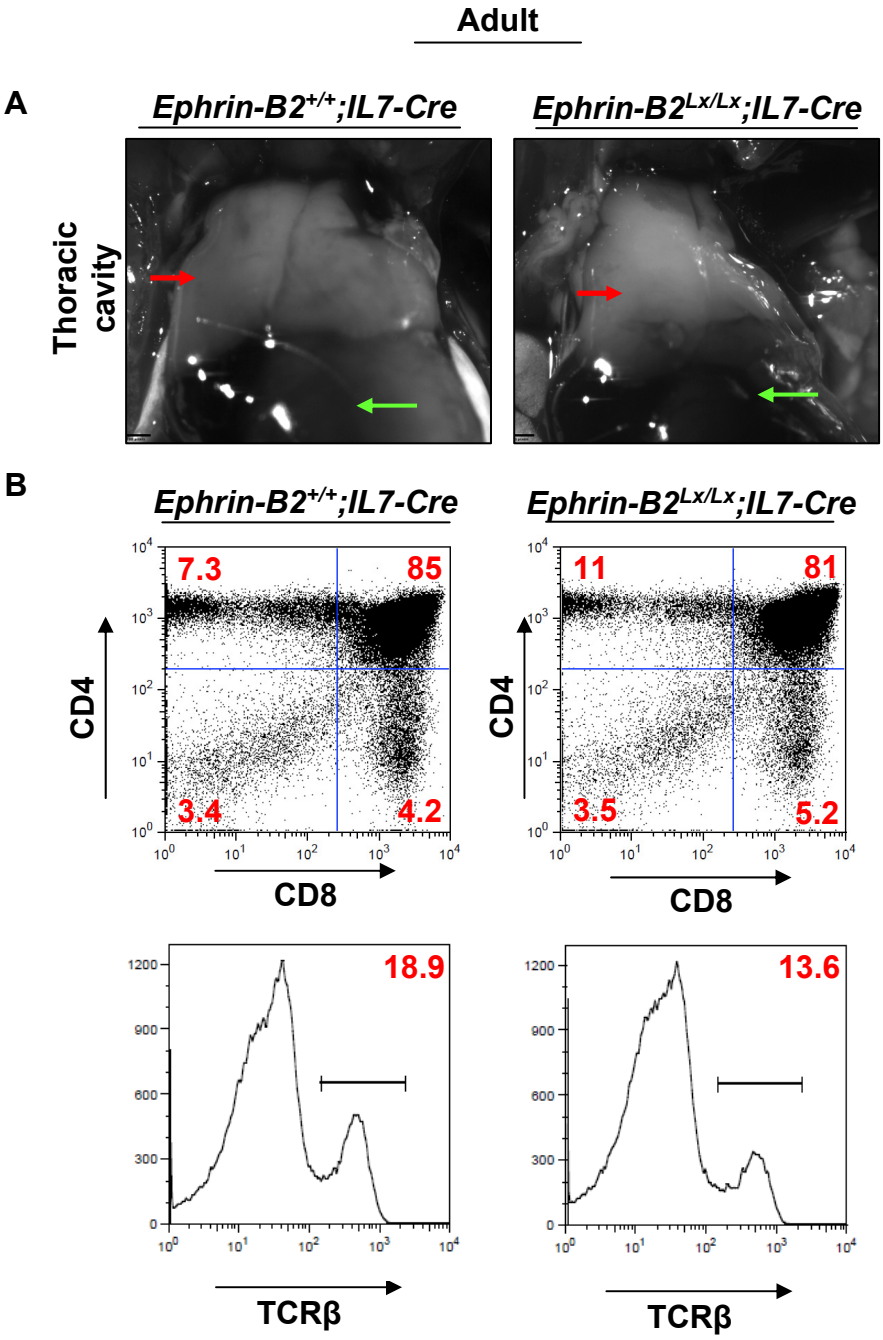

E15.5
A
CD45+
eYFP+
CD45-YFP-
2.2%
98.7%
83.4%
ephrin-B2
B
CD45+
eYFP+
CD45-YFP-
14.4%
99.5%
94.8%
EphB4

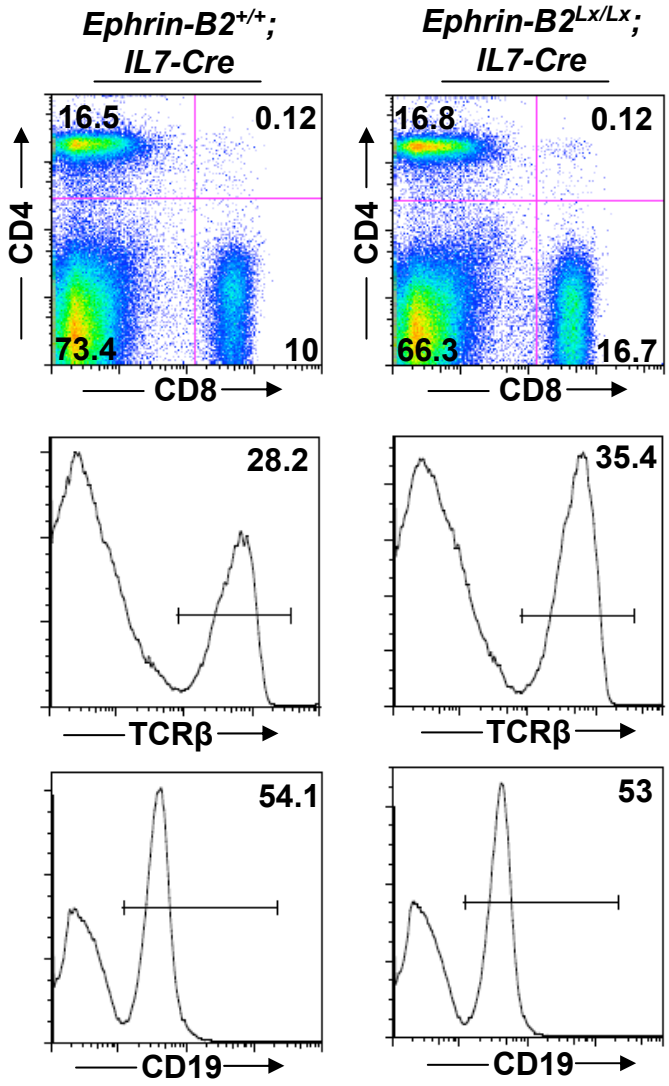
A

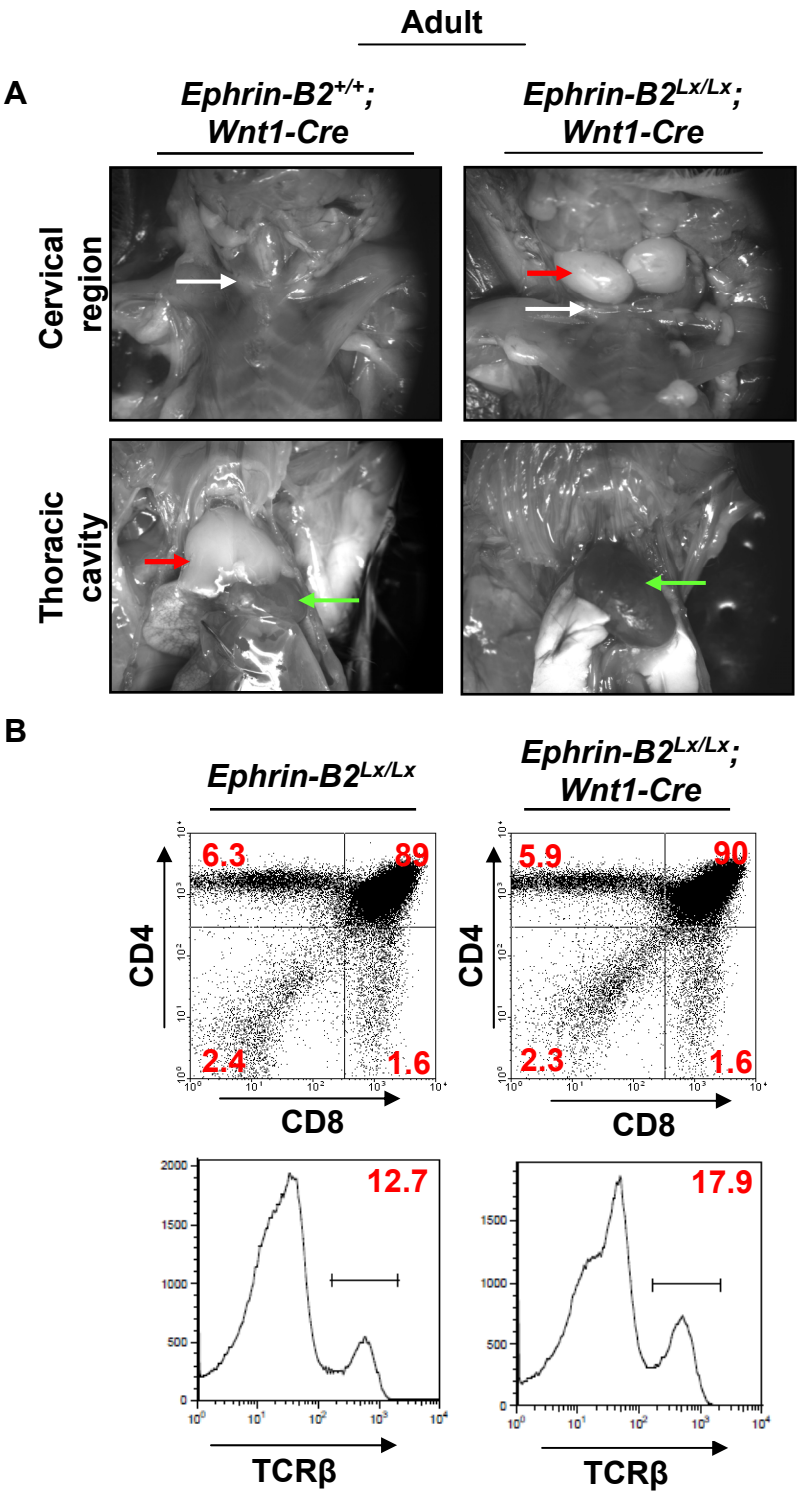

ephrin-B2Lx/Lx
ATG
H
H
H
LoxP
LoxP
neo
FRT
FRT

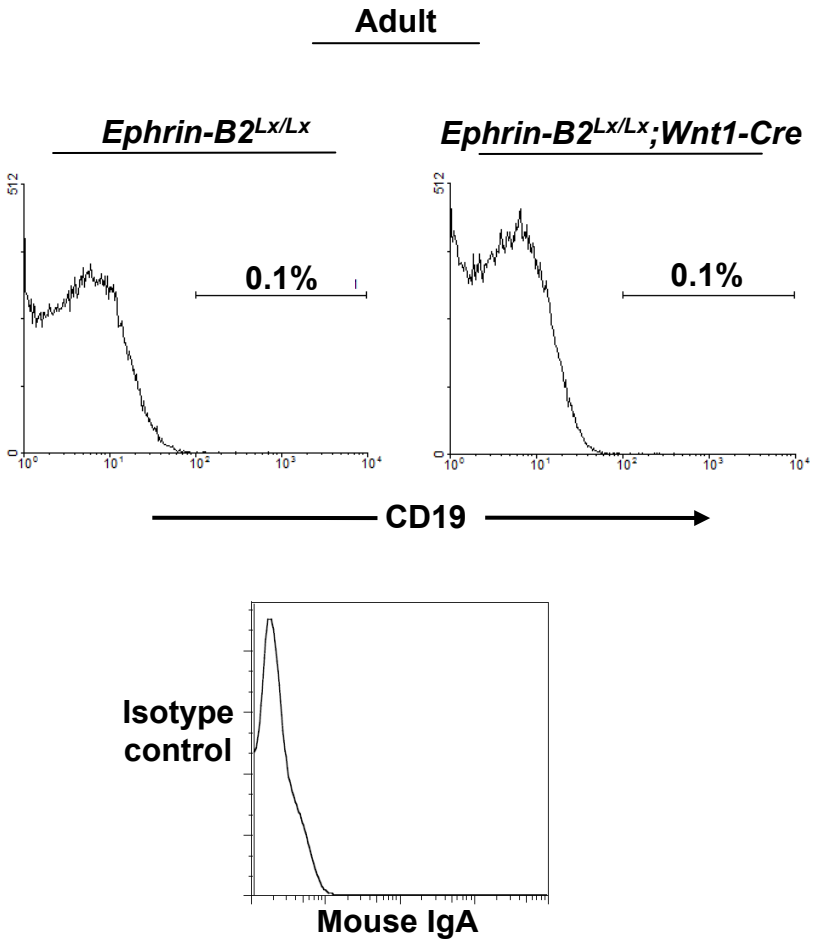
B

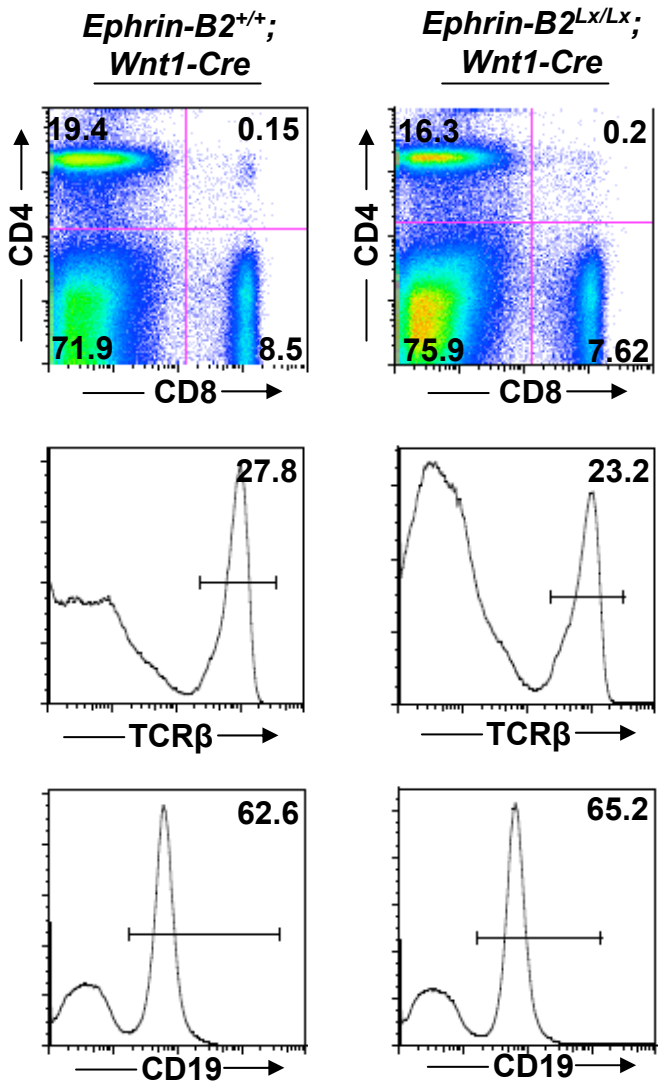

IL-7 Cre
IL-7 Locus
5' UTR
Intron 1
a
b
a
Cre
Zeo
b
FRT
FRT

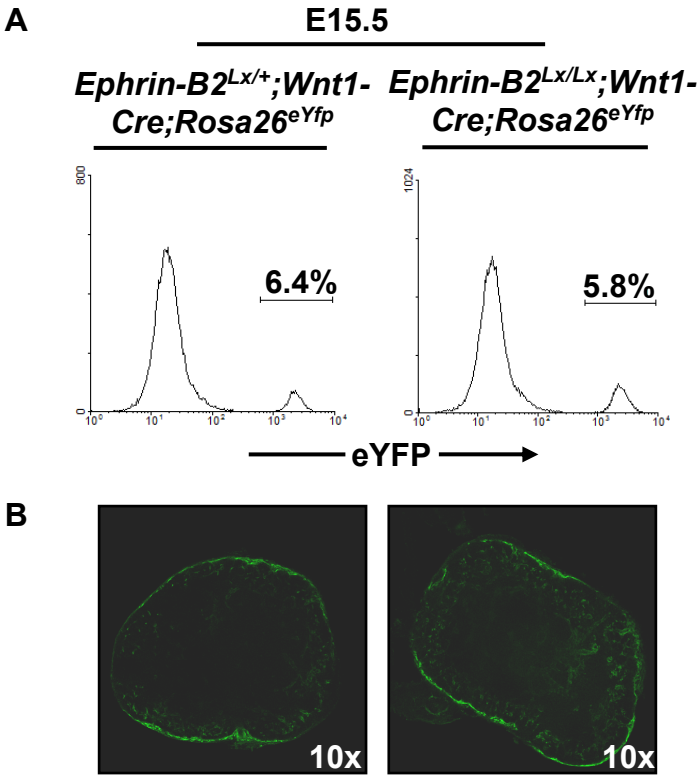

Adult
A
Ephrin-B2+/+;IL7-Cre
Ephrin-B2Lx/Lx;IL7-Cre
Thoracic cavity
B
Ephrin-B2+/+;IL7-Cre
Ephrin-B2Lx/Lx;IL7-Cre
CD4
CD8
7.3
85
3.4
4.2
11
81
3.5
5.2
18.9
13.6
TCRβ


Ephrin-B2+/+; IL7-Cre
Ephrin-B2Lx/Lx; IL7-Cre
16.5
0.12
73.4
10
16.8
0.12
66.3
16.7
CD4
CD8
28.2
35.4
TCRβ
54.1
53
CD19


Adult
A
Ephrin-B2+/+; Wnt1-Cre
Ephrin-B2Lx/Lx; Wnt1-Cre
Cervical region
Thoracic cavity
B
Ephrin-B2Lx/Lx
Ephrin-B2Lx/Lx; Wnt1-Cre
6.3
89
2.4
1.6
5.9
90
2.3
1.6
CD4
CD8
12.7
17.9
TCRβ


Adult
Ephrin-B2Lx/Lx
Ephrin-B2Lx/Lx;Wnt1-Cre
512
0
0.1%
0.1%
CD19
Isotype control
Mouse IgA


Ephrin-B2+/+; Wnt1-Cre
Ephrin-B2Lx/Lx; Wnt1-Cre
19.4
0.15
71.9
8.5
CD4
CD8
16.3
0.2
75.9
7.62
CD4
CD8
27.8
TCRβ
23.2
TCRβ
62.6
CD19
65.2
CD19

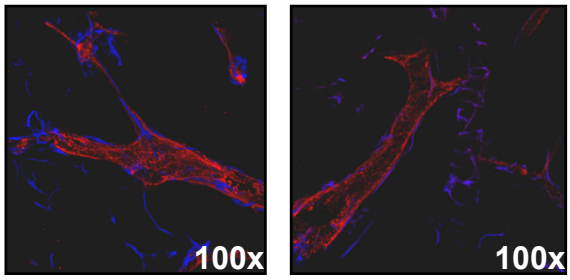

A
E15.5
Ephrin-B2Lx/+;Wnt1-Cre;Rosa26eYfp
Ephrin-B2Lx/Lx;Wnt1-Cre;Rosa26eYfp
800
1024
6.4%
5.8%
eYFP
B
10x
10x

A

Adult

Ephrin-B2$^{Lx/Lx}$

Ephrin-B2$^{Lx/Lx}$;Wnt1-Cre;Rosa26^{eYfp}

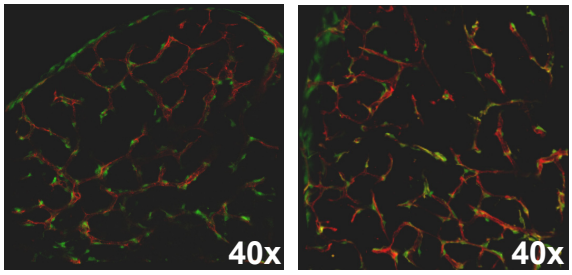


Endomucin Desmin

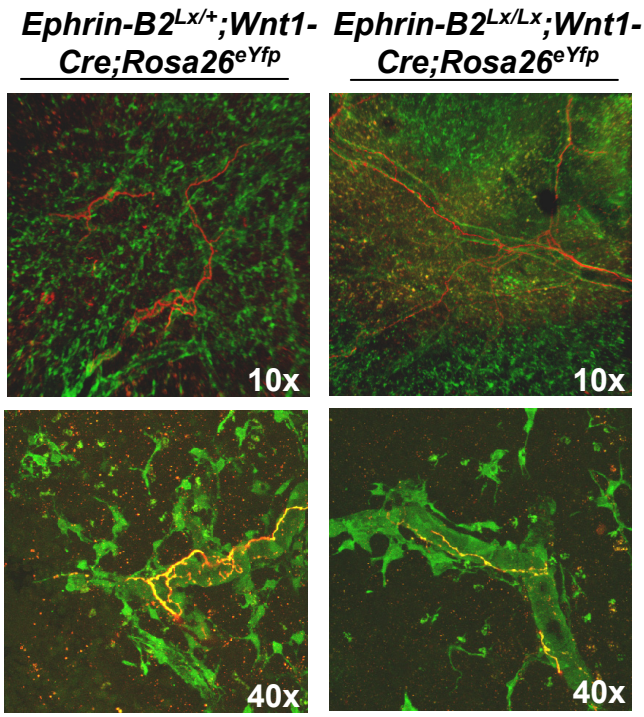
B

Wnt1-Cre; Rosa26^{eYfp}

Ephrin-B2$^{Lx/Lx}$;Wnt1-Cre;Rosa26^{eYfp}



eYFP Endomucin


Ephrin-B2Lx/+;Wnt1-Cre;Rosa26eYfp
Ephrin-B2Lx/Lx;Wnt1-Cre;Rosa26eYfp
10x
10x
40x
40x
TUJ1 eYFP

Chapter Six: What is the mechanism of thymus migration into the thoracic cavity during organogenesis?

In mice with ephrin-B2 specifically deleted on NCCs, the thymic rudiment detaches normally from the pharynx, but there is delayed separation of the combined thymus and parathyroid domains. Moreover, the migratory behaviour of individual NCCs is abnormal in these mice. Thus, it is proposed that NC derived cells that surround and invade the thymic rudiment direct the migration of the thymus by a process of collective cell migration. This mechanism requires intrinsic signals from the NCCs, combined with environmental signals from the surrounding tissues to move the organ from the cervical region into the thoracic cavity.

6.1 Experimental Rationale

The mechanisms that control the various morphogenetic events during early thymus organogenesis are still largely unknown, although some genetic pathways have been identified that affect these processes. The Hoxa3 and Pax1/9 transcription factors are required for proper separation of the developing thymic and parathyroid primordia from the pharynx, and may also contribute to their separation from the pharynx or each other, although the cell-type-specificity of these functions is poorly understood (5, 21, 22). *Splotch* embryos, which have a null allele of Pax3 and are largely deficient of NCCs, also exhibit pharyngeal pouch defects including an ectopic thymus (23, 24). This NCC deficiency resulted in delayed separation of the thymus and parathyroid from the pharynx, and the boundary between thymus and parathyroid-fated domains was abnormal. These results strongly implicated that NCC migration to the third pharyngeal pouch is required for patterning and morphogenesis of the thymus and parathyroids (23). Interestingly, embryos which have deleted the TGF-β type-1 receptor, ALK5, in NC derived cells also have ectopically located thymi as a result of delayed separation of the parathyroid and thymic rudiments without histological or other differentiation defects. Moreover, this latter defect was not due to impaired NC migration to the third pharyngeal pouch, but was thought to be due to increased apoptosis in post-migratory NCCs (25).

In the previous chapter, it was shown that the thymus is also ectopic in mice deficient in ephrin-B2 on NCCs. This could be due to defects that occur during initiation, patterning, separation or migration of the thymus. In order to determine how ephrin-B2 affects thymus development, it was necessary to examine the processes of separation and migration in mice with ectopic thymi.

6.2 Experimental Strategy

To investigate the mechanism by which the thymus, which originates at the pharyngeal pouches and migrates into the thoracic cavity, a similar strategy to that described in chapter five was used. Thus, mice with LoxP sites flanking exon 2 of the *Efnb2* gene (202) were crossed with mice expressing Cre recombinase in NCCs under the control of *Wnt1* (198) promoter and regulatory elements. Cre activity was reported via activation of a 'silent' enhanced yellow fluorescent protein (eYFP), expressed from the Rosa26 locus (201).

The ectopic location of the thymus could result from defective separation of the thymic rudiment from the pharynx and/or from the parathyroid. In order to determine whether separation from the pharynx and parathyroid occurs at the normal time in the mutant mice described here, H&E stained sections through the cervical and thoracic regions were examined from E12.5 and

E13.5 *Wnt1-Cre;Rosa26*^{eYfp} and *Ephrin-B2*$^{Lx/Lx}$*;Wnt1-Cre;Rosa26*^{eYfp} embryos. Moreover, it was also possible that delayed or abnormal separation might result from defects in the formation of the boundary between thymus and parathyroid domains. In order to investigate whether there is mixing of thymus and parathyroid fated cells in the mutant mice described here, an *in situ* hybridisation analysis of genes associated with thymus (FoxN1) and parathyroid (GCM2) were carried out on E11.5 sections from mutant and wild type mice.

In a second set of experiments, the movements of single cells, and whole thymi, were assessed *in vitro.* Thus, thymi from E13.5 *Wnt1-Cre;Rosa26*^{eYfp} and *Ephrin-B2*$^{Lx/Lx}$*;Wnt1-Cre;Rosa26*^{eYfp} embryos were enzymatically digested and left to attach onto glass-bottom culture dishes. Additionally, whole thymic rudiments were embedded in a collagen gel and cultured for 48 hours. EphB4-Fc and ephrin-B2-Fc cross linked with goat anti-human IgG, and control Fc cross linked with IgG, were added to the culture medium in order to block or stimulate signalling artificially. The Fc chimeric proteins consist of the extracellular domain of either EphB4 or ephrin-B2, fused to the carboxy-terminal 6X histidine-tagged Fc region of human IgG. When added to the culture medium, EphB4-Fc binds to ephrin-B2 ligands expressed on the cell surface. Binding of EphB4-Fc to ephrin-B2 ligands on the cell surface blocks signalling downstream of the ephrin ligand (reverse signalling). When cross linked with IgG, ephrin-B2-Fc mimics a cell surface bound ligand and

is able to induce forward signalling through the receptor with which it binds. Thus, it is possible to investigate the signalling pathways activated by each element.

6.3 Results

6.3.1 Deletion of ephrin-B2 does not disrupt separation of the thymus/parathyroid rudiment from the third pharyngeal pouch.

The ectopic thymus location in the mutant mice described here could be the result of a delay in the separation of the organ primordia from the pharynx, and subsequent inability of the thymus rudiment to reach the superior mediastinum before the thoracic cavity closes. Since Eph/ephrins have been shown to play a role in boundary formation and maintenance between discrete structures in other organs and tissues, it is possible that they could also be involved in this process. The combined thymus and parathyroid primordium, which starts forming at E11 detaches from the third pouch endodermal epithelium by E12. In order to assess whether the defect seen in *Ephrin-B2$^{Lx/Lx}$;Wnt1-Cre* mice is due to a failure in forming a boundary between the combined rudiment and the pharyngeal pouch leading to delayed separation, paraffin embedded E12.5 and E13.5 control *Ephrin-B2$^{+/+}$* and *Ephrin-B2$^{Lx/Lx}$;Wnt1-Cre;Rosa26^{eYfp}* embryos were sectioned and stained with haematoxylin and eosin (Figure 6.1A & B). Analysis of serial

sections through the neck and thoracic cavity revealed that the combined rudiment does separate fully from the third pharyngeal pouch with normal timing (Figure 6.1A & B). These experiments were performed in collaboration with Julie Gordon, University of Georgia, USA.

In addition to separating from the third pharyngeal pouch, the combined thymus and parathyroid rudiments also divide from each other into two separate structures; the thymus migrates into the thoracic cavity, whereas the parathyroid remains in the neck adjacent to the thyroid gland. In order to determine whether deletion of ephrin-B2 on NCCs disrupts the separation of the thymus and parathyroid rudiment from each other, serial sections of the cervical and thoracic regions were examined. H&E-stained transverse sections through the neck and thoracic regions of E12.5-13.5 mutant embryos showed a defect in separation of the thymus and parathyroid domains from each other when compared to control embryos (Figure 6.2). This phenotype was due to a delay, rather than failure, since dissection of adult mutant mice showed that the thymus and parathyroid eventually separate from each other. To investigate whether this delayed organ separation was due disruption of the boundary between thymus and parathyroid, *in situ* hybridisation for *Foxn1* (thymus, Figure 6.3, right) and *Gcm2* (parathyroid, Figure 6.3, left) was carried out on sagittal sections of E11.5 wild type *Ephrin-B2*$^{+/+}$ and mutant *Ephrin-B2*$^{Lx/Lx}$*;Wnt1-Cre* embryos. There was no mixing of parathyroid and thymus cells in the absence of

ephrin-B2 expression on NCCs (Figure 6.3, bottom) when compared to the wild type control (Figure 6.3, top). These experiments were performed in collaboration with Julie Gordon, University of Georgia, USA.

Together, these results indicate that deletion of ephrin-B2 from NC derived cells does not affect or delay the separation of the thymus/parathyroid combined rudiment from the third pharyngeal pouch, but it does affect the separation of the thymus and parathyroid rudiments from each other.

6.3.2 Forward signalling through Eph receptors is required for normal thymus positioning

In addition to the forward signalling through Eph receptors, the ephrin-B ligands themselves are also able to signal; this is known as reverse signalling. Ablation of the receptor or ligand blocks both forward and reverse pathways. However, it is possible to block either the forward or reverse pathway without additionally blocking the reciprocal pathway, by mutating protein sequences that code for one or more of the signalling domain of the receptor or ligand. For example, the intracellular domain of ephrin-B2 has five conserved tyrosine residues and a PDZ domain which mediate two reverse signalling pathways. Mice lacking the c-terminal PDZ interaction site (*ephrin-B2*$^{\Delta V/\Delta V}$) exhibit major defects in the lymphatic system and problems in vascular development and are neonatal lethal; mice lacking the five

conserved tyrosine residues (*ephrin-B2*$^{5Y/5Y}$) only have a very minor phenotype (203). The phenotypic differences between *ephrin-B2*$^{\Delta V/\Delta V}$, which blocks only the reverse signalling pathway, and *ephrin-B2*$^{-/-}$, which blocks both pathways, are probably a result of the different pathways that are activated downstream of the receptor and ligand. In order to determine whether forward signalling or reverse signalling is responsible for thymus migration, the two different ephrin-B2 signalling mutants, *ephrin-B2*$^{\Delta V/\Delta V}$ and *ephrin-B2*$^{5Y/5Y}$ were examined. In *ephrin-B2*$^{\Delta V/\Delta V}$ and *ephrin-B2*$^{5Y/5Y}$ mutants the thymus was normally sited, thus, the signalling domains disrupted in these mutants appear to be not required for correct thymus migration (Figure 6.4). These results provide some but by no means definitive proof that reverse signalling may not be involved in the proper positioning of the thymus and suggesting that forward signalling plays the dominant role in this process.

6.3.3 Eph/ephrin signalling is required for normal migration of NC-derived mesenchymal cells

The normal separation of the combined rudiment from the pharynx suggested that thymus migration itself may be delayed. To examine whether ephrin-B2 expression on NCCs is involved in their mobility, E13.5 mutant *Ephrin-B2*$^{Lx/Lx}$*;Wnt1-Cre;Rosa26*^{eYfp} and control *Ephrin-B2*$^{+/+}$*;Wnt1-Cre;Rosa26*^{eYfp} embryonic thymi were digested with collagenase and the

resulting cell suspension was incubated in glass-bottom culture dishes overnight. The next day $eYFP^+$ cell movements were imaged by time-lapse widefield microscopy over a period of 4 hours. Deletion of ephrin-B2 resulted in marked changes in the cells' motile behaviour. Mutant cells appeared to form many unstable lamellipodial protrusions, were unable to retract these protrusions, and had frequent changes in overall direction of movement (Figure 6.5B). Tracking these cells indicated a displacement of 6.4μm over four hours (Figure 6.5E) and a low meandering index (a measure of directionality where 1 = migration in one direction, and 0 = totally random movement) of 0.19 (Figure 6.5F). In contrast, control cells appeared polarised, migrated to a total displacement of 45.5μm over four hours and exhibited a meandering index of 0.67 (Figure 6.5A, E & F).

It was possible to induce control cell behaviour similar to that seen in the mutants using an EphB4 fusion protein that blocks signalling from this receptor. Under these conditions control cells became unable to polarise, acquired many unstable lamellipodial protrusions, exhibited a displacement of 5.89μm over four hours and a decreased meandering index of 0.16 (Figure 6.5C, E & F). In contrast, incubation of mutant cells with an ephrin-B2 fusion protein cross linked with IgG, which binds and activates signalling by the EphB receptors, restored polarisation and directional migration. Thus the cells showed a 23.6μm displacement over four hours and an increased meandering index of 0.55 (Figure 6.5D, E & F). It is possible that this is the

result of a cell autonomous function, or could be due to either previous cell-cell interactions or cross talk with other receptor signalling pathways in the same cell.

6.3.4 Analysis of whole organ cultures indicate that the thymus may relocate into the thoracic cavity by collective cell migration

The migration of individual NCCs may direct the relocation of the thymus into the thoracic by a process of collective cell migration. This mechanism requires that the cells within the organ are tightly adhered to each other whilst those at the leading edge migrate forward, dragging with it the rest of the rudiment. This would suggest that there is an overall polarity of the thymus, with NCCs at the front behaving differently to those at the back. In order to examine this possibility whole thymic rudiments from E13.5 mutant *Ephrin-B2*$^{Lx/Lx}$*;Wnt1-Cre;Rosa26*^{eYfp} and control *Ephrin-B2*$^{+/+}$*;Wnt1-Cre;Rosa26*^{eYfp} embryos were embedded into a collagen gel that mimics the three-dimensional structure of living tissues. Explants were cultured in the gels for 48 hours, after which they were fixed with paraformaldehyde and stained with an antibody detecting eYFP.

NC derived cells from control thymi appear to migrate away from the rudiment preferentially towards one axis (Figure 6.6, left). Moreover, a small fragment of thymic tissue appears to have been dragged away from the

rudiment in the same direction as the migrating NCCs (Figure 6.6, top left, red arrow). Fragments of thymus tissue that appear to have broken off from the main thymic rudiment were observed in five out of eight control rudiments examined. In contrast, fewer NCCs appear to migrate away from ectopic thymi and they exit equally at all axes of the rudiment (Figure 6.6, right). In addition, thymic tissue did not detach and move away from any of the three ectopic rudiments examined.

Together these results indicate that the motility of NCCs is disrupted in the absence of ephrin-B2, and this may correlate with defective migration of the whole thymic rudiment. Whole organ cultures of wild type and ectopic thymi suggest that there may be an inherent polarity to the thymic rudiment, which is determined by the NCCs and that loss of ephrin-B2 prevents the NCCs from their role in thymus migration.

6.4 Discussion

6.4.1 Separation or Migration; which is defective in mice with ephrin-B2 deficient NCCs?

As discussed in chapter five, ectopia of the thymus can result from defects in a number of stages of thymus organogenesis, from initial patterning of the pharyngeal pouch to migration of the rudiment. In the mutants described

here, initiation of organ formation is apparently normal since functional thymi and parathyroid epithelium develop. Moreover, NCCs contributed normally to ectopic thymic in mice deficient for ephrin-B2 expression on NC derived cells, thus, the defect in organ location may result from impaired separation from the pharynx and/or parathyroid, or migration into the thoracic cavity.

In order for the thymus to migrate into the thoracic cavity it must be released from the pharynx. A number of mutants have been described that have defects in separation of the combined rudiment from the pharynx. In contrast, the mutants described here do not exhibit a defect in separation from the pharynx, although they do remain attached to the parathyroid for a longer time than in control embryos. The defect in thymus-parathyroid separation is a delay rather than complete block, since in the adult they are no longer joined but are discrete organs. However, the delay in separation may hold the thymic rudiment in the cervical region long enough for the thoracic cavity to close so that the thymus can no longer migrate caudally and ventrally. It is not clear why there is a difference in the mechanism of separation from the pharynx than from the parathyroid. One possibility may be that release from the pharynx is an active process controlled by the NCCs surrounding the capsule, but separation from the parathyroid is a passive process that requires the migration of the thymus away from the parathyroid domain. Support for this hypothesis was provided by the finding that motility of NC derived cells surrounding the rudiment was defective.

Mutants with ectopic thymi have been described that result from an abnormal boundary between the thymus- and parathyroid-domains of the common primordium (23). The authors suggested that a decrease in the number of NCCs was the cause of the irregular boundary, and this prevented the separation of the two organs. Eph/ephrins expression often demarcates boundaries, such as in the hindbrain and on veins and arteries, suggestive of a mechanism of repulsion between Eph and ephrin-expressing cells. Thus, it is possible that repulsion of thymus- and parathyroid-specific cells that express different Eph or ephrin molecules drives them into distinct domains, creating a tight boundary between the two rudiments. However, in contrast to previously published reports showing mixing between the two domains, the mutants described here do not exhibit intermingling of FoxN1- and GCM2-expressing cells. These results would support the hypothesis that the delay in division of the rudiments is not actually a separation defect, instead, a lack of migration pulling the two organs apart.

Since the parathyroid and thymus rudiments develop together from a combined rudiment, the parathyroid might also exhibit location defects. Moreover, it has been suggested that the parathyroids are pulled with the thymus as it migrates caudally and ventrally, but they detach from the thymus and are left in the cervical region near the thyroid. The function of the parathyroid does not appear to be affected by loss of ephrin-B2 on NCCs,

since the mice do not exhibit defects relating to its role as an endocrine gland. Nevertheless, it would be interesting to determine the position, and the contribution of the NC, of the parathyroid in the mutant mice described here.

6.4.2 Ectopic thymi are not equivalent to the cervical thymus

The existence of cervical thymi was recently reported in the mouse. The cervical thymi only expand after birth, but are known to support T cell development and can correct T cell deficiencies in athymic nude mice upon transplantation (258, 259). Since the ectopic thymi described here are found in a similar location as the cervical thymi reported in a few mouse strains, it is tempting to dismiss their existence as merely enlarged cervical thymi. However, there are a number of reasons why this is unlikely. The cervical thymus expands after birth, probably from fragments of thymic tissue that break off from the rudiment as it descends into the thoracic cavity. In contrast, the ectopic thymi are present in the cervical region from mid-gestation. Moreover, although the cervical organ has a typical medulla-cortex region, it consists of just one lobule and is much smaller than the thoracic and ectopic thymi. This is not the case in the thoracic and ectopic thymi, where both consist of many lobules that each harbour medullary and cortical regions, and both thoracic and ectopic thymi exhibited very similar cellularity. In addition, there were subtle structural changes in the cervical

thymus that are not apparent in the ectopic organs described here. Thus, the mesenchymal fibroblastic compartment was less integrated with the mTECs when compared to the thoracic thymus.

It is possible that there are similar deficiencies in cell migration in mice with ectopic thymi and those with cervical thymi. For this reason, it would be interesting to investigate the cause of cervical thymi and why they only arise in certain genetic backgrounds. To do this, it may be useful to compare the expression of Eph/ephrin molecules on NCCs from ectopic and cervical thymi. In addition, the mobility of NCCs from cervical thymi could be assessed by a similar method to that described here.

6.4.3 Forward signalling through Eph receptors appears to direct thymus migration

Signalling can occur downstream of both Eph receptors and ephrin ligands. Ablation of either the receptor or the ligand disrupts both forward and reverse pathways, making it difficult to establish whether molecules downstream of the Eph receptor or ephrin ligands are responsible for the normal positioning of the thymus. However, ephrin-B signalling mutants, which only block reverse signalling by disrupting the PDZ domain or phosphotyrosine docking sites of ephrin-B2 showed no disruption in thymus

location, indicating that forward signalling through EphB receptors is probably responsible for the observed phenotype.

Neither of the ephrin-B2 signalling mutants reflected a complete block in reverse signalling through the ligand, since in one case the PDZ domain is disrupted leaving the phosphotyrosine docking sites intact and in the other the phosphotyrosine docking sites were mutated whilst the PDZ domain was still functional. Thus, there may be biological activity elsewhere in the transmembrane ligand. In support for the role of EphB receptors in the mechanism of thymus migration were experiments using cross linked ephrin-B2/Fc chimeric proteins to activate forward signalling and rescue the migration defect of individual cells. However, there may be crosstalk with other signalling pathways that are blocked or activated using this technique. Thus, it will be necessary to examine ephrin-B2 and EphB4 signalling mutants that completely block either the reverse or forward signalling pathways to define which pathway is responsible for thymus migration.

Signalling molecules downstream of Eph receptors include small GTPases of the Rho and Ras families, focal adhesion kinase, the JAK/STAT and PI3K pathways (261, 262). Rho family molecules such as Rac mediate actin dynamics, which controls cell shape and movement by promoting the formation of lamellipodia, filopodia and stress fibres (143, 144, 263). *In vitro* experiments described here demonstrate that in the absence of ephrin-B2

expression, polarisation and migration of NC derived cells is defective. This phenotype was rescued by exposure of the mutant cells to soluble ephrin-B2/Fc cross-linked with IgG, thus providing a substitute ligand to activate the receptor. Together, these results strongly suggest that signalling downstream of EphB receptors is required for normal mobility of thymic mesenchyme and for correct positioning of the thymus. To test this hypothesis, embryos with NCCs deficient for Rac1 were examined. However, due to the role of Rac1 signalling elsewhere in development, the embryos died at E12 from severe cardiac defects and other malformations. Thus, it was not possible to examine the thymus of these mice.

6.4.4 Migration of NCCs is impaired in the absence of ephrin-B2

In order to assess a potential defect in the migration of the organ, the mobility of NCCs deficient in ephrin-B2 was examined. Similar to experiments that showed that smooth muscle cells were defective in spreading, focal-adhesion formation and polarised migration in the absence of ephrin-B2, it was found that the NCCs were likewise affected. Thus, they were unable to polarise and exhibited a greatly reduced displacement. Foo and colleagues suggested that impaired motility caused defective mural-cell coverage of the vasculature (179). Likewise, it is possible that impaired mobility of NC derived cells, which surround the capsule of the thymic

rudiment, causes a failure in the migration of the organ into the thoracic cavity.

It is unclear as to why NCCs are able to migrate into the pharyngeal pouches, but once associated with the thymus they then exhibit impaired mobility. It is possible that there are two different mechanisms, and thus different molecules, directing migration at these two different stages. In which case, migration from the neural tube to the pharyngeal pouch involves the migration of single cells that is independent of ephrin-B2 expression. In contrast, collective migration of the whole organ, which requires tightly adhered NC derived cells surrounding the rudiment to co-ordinate the movement of the organ is dependent upon the expression of ephrin-B2.

It is possible that the behaviour of NCCs is different in culture than *in vivo*, since two dimensional cultures may not provide an accurate representation of the environment of living tissues. To investigate this possibility, and to definitively answer the question as to whether separation or migration is impaired in the mutant mice described here, it might be possible to observe thymus development in vivo. Using multi-photon microscopy to image live embryos that have NCCs marked with eYFP, it may be possible to examine the processes of separation and migration of the thymic rudiment as they occur. Alternatively, dissection and culturing of the whole pharyngeal pouch, including the combined thymus- and parathyroid-rudiment at E11.5-E12, in a

collagen gel may provide similar answers. The three-dimensional structure of the collagen gel would provide an alternative, more physiological, environment for the rudiment to migrate through (264). If a delay in the separation of the rudiment from the parathyroid and subsequent closure of the thoracic cavity does prevent thymus migration, the thymic rudiment would continue to migrate in the collagen gel in response to a chemoattractant, such as FGF ligands. However, if the rudiments do not migrate in comparison to wild type thymi, this would indicate that migration rather than separation defects were the cause of ectopia.

6.4.5 Collective cell migration of the thymic rudiment

The data presented here raises an interesting question; How does the mobility of individual cells influence the migration of the whole thymus? There are precedents for the movement of cell groups, sheets, or strands consisting of multiple cells that are mobile yet simultaneously connected by cell-cell junctions elsewhere in embryogenesis (265, 266). During morphogenesis a number of developmental steps require collective cell migration; cells of the inner blastocyst (267); epithelial sprouts (268); migration of drosophila border cells (269) and movement of epithelial cells at the rim of the otic and thyroid placodes (270). This process requires that the migrating mass comprising of cells that are tightly adhered to each other by cell-cell junctions is dragged forward by the cells at the leading edge, whilst

the relative position of the cells within the group is maintained (271). Thus, it is possible that signalling downstream of ephrin-B2-EphB interactions contributes to the formation of lamellipodia by NC derived cells, which protrude into the surrounding extracellular matrix. Subsequent shortening of actin filaments then results in retraction of the trailing edge and movement of the cell body, which drags behind it the whole rudiment.

In support of this hypothesis are experiments in which whole thymic rudiments were cultured in a three dimensional collagen gel. After 48 hours, NCCs leave the organ preferentially at one axis of the control rudiments, and in some cases it appears that a piece of thymus detaches and is dragged away. In ectopic rudiments, a much reduced number of NCCs appear to leave the organ and they appear to exit at all axes. It is important to note that these results may be an artefact of the experimental technique, thus examining development of the thymus *in situ* is needed to reveal the answers to these questions.

A mechanism by which collective cell migration drives the relocation of the thymic rudiment would suggest that the organ has an inherent polarity. Thus, the NCCs at the leading edge may be somehow different to those at the back, perhaps in the expression of certain signalling molecules or receptors. In the migrating lateral line of zebrafish, Wnt, β-catenin and FGF signalling control primordium polarity by differential regulation of gene expression in

the leading versus trailing edge. Moreover, blocking FGF signalling with a biochemical inhibitor prior to organ migration leads to stalling. It was shown that Wnt and β-catenin signalling is activated only in leading cells, which leads to the production of an inhibitor that confines activation of this pathway to the leading zone (272). A similar mechanism, or others, may exist to establish and maintain polarity of the thymic rudiment. In order to determine the validity of this hypothesis, it will be necessary to examine the expression of genes, including FGFs, Wnt and β-catenin in the NCCs of E12.5-E13.5 thymi by whole organ immunohistochemistry.

6.4.6 Summary

The ectopic thymi from mice with ephrin-B2 deficient NCCs was examined, and shows that, unlike other mutants with ectopia described previously, this does not result from impaired separation from the pharynx. However, separation of the thymus from the parathyroid is delayed, thus there are two possibilities for the ectopic location of the thymus; either the delay in separation from the parathyroid that holds the thymus in the cervical region, or, a there is a defect in organ migration. The latter mechanism is more likely, since the migration of individual NCCs is blocked in the ectopic thymus. Thus, a mechanism of collective cell migration is proposed, directed by molecules downstream of the Eph receptor on NCCs, for the relocation of the thymus into the thoracic cavity.

Figure 6.1: The combined thymus and parathyroid rudiment detaches normally from the pharynx. A&B Histological analyses of cervical region in E12.5 and E13.5 wild type (*Ephrin-B2*$^{+/+}$, top) and mutant (*Ephrin-B2*$^{Lx/Lx}$*;Wnt1-Cre;Rosa26*^{eYfp}, bottom) embryos. **A** Transverse sections through E12.5 wild type (top left; upper cervical region, top right; lower cervical region), and mutant embryos (bottom left; upper cervical region, bottom right; lower cervical region). Top and bottom panels are at equivalent positions along the anterior-posterior axis, thus left panels are higher in the cervical region than right panels, indicating the slightly more anterior location of the mutant thymus lobes. * in top left panel indicates the area corresponding the location of the mutant thymus lobe in bottom left panel. **B** Transverse sections through E13.5 wild type (top left; cervical region, top right; thoracic region), and mutant embryos (bottom left; cervical region, bottom right; thoracic region). Top and bottom panels are at equivalent positions along the anterior-posterior axis, again indicating the significantly more anterior location of the mutant thymus lobes. * in top left panel indicates the equivalent positions of the mutant thymus lobes in bottom left panel. * in bottom right panel indicates the equivalent position of the normal thymus in top right panel. **th**, thymus. Scale bar = 100μm. This experiment was performed in collaboration with Julie Gordon, University of Georgia.

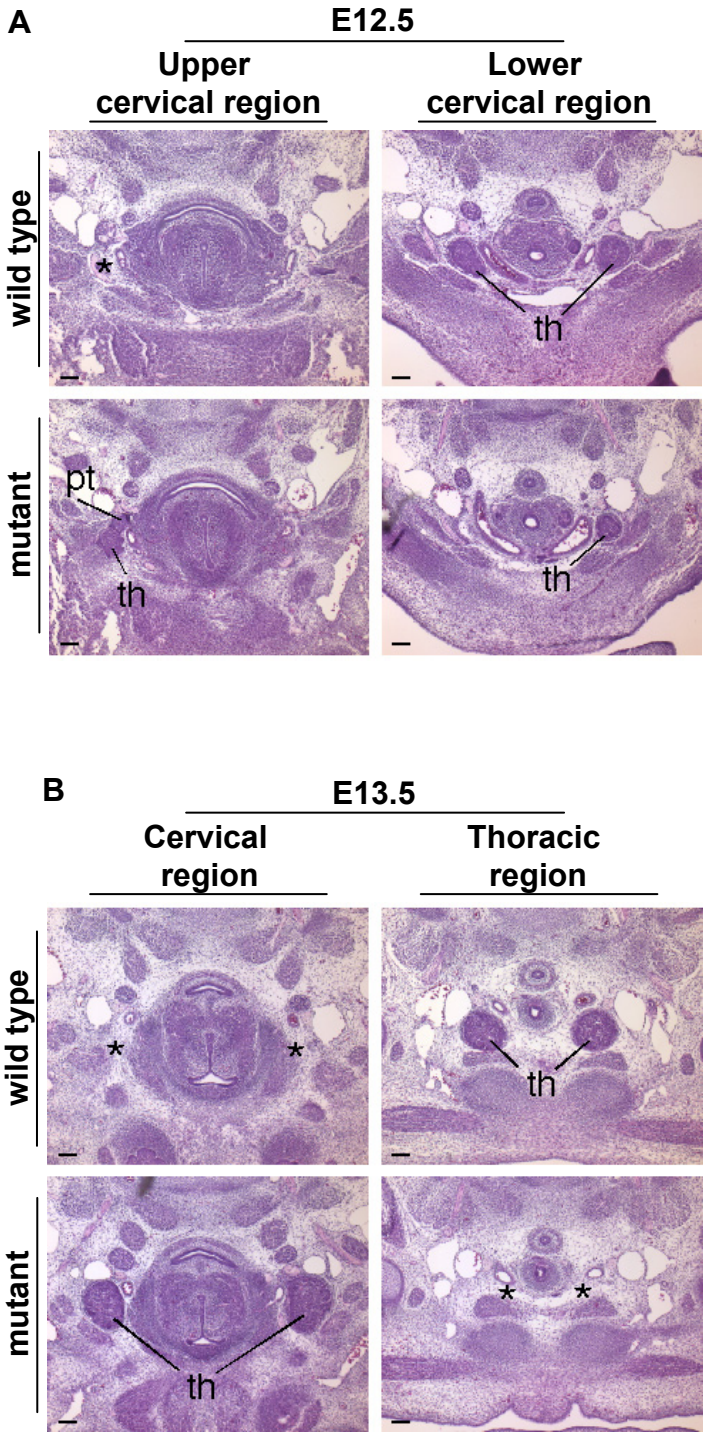
Figure 6.2: Separation of the thymus and parathyroid is delayed in ectopic thymi. Histological analyses of cervical region in E12.5 (left) and E13.5 (right) wild type (*Ephrin-B2*$^{+/+}$, top) and mutant (*Ephrin-B2*$^{Lx/Lx}$*;Wnt1-Cre;Rosa26*^{eYfp}, bottom) embryos, indicating parathyroid position. Top panels indicate the normal location of the parathyroids, attached to the thymus at E12.5 (left) and detached from the thymus and adjacent to the thyroid gland at E13.5 (right). In mutant embryos (bottom), the parathyroids remain attached to the thymus lobes at both E12.5 (left) and E13.5 (right). **th**, thymus. **pt**, parathyroid. **ty**, thyroid. Scale bar = 100μm. This experiment was performed in collaboration with Julie Gordon, University of Georgia.

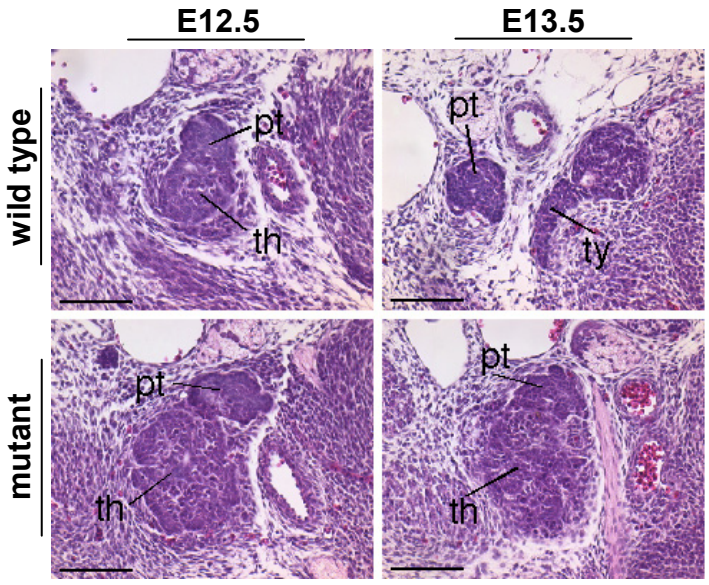
Figure 6.3: Patterning of thymus and parathyroid domains is normal in mice with ectopic thymi. Sagittal sections through the common thymus-parathyroid primordium at E11.5 showing prospective organ domains, marked by *Foxn1* (thymus, left) and *Gcm2* (parathyroid, right). Top panels; adjacent sections through wild type primordium, Bottom panels; adjacent sections through mutant primordium. Dotted line in left panels indicate thymic rudiment, and in right panels, parathyroid rudiment. Scale bars = 100μm. This experiment was performed in collaboration with Julie Gordon, University of Georgia.

Figure 6.4. Reverse signalling through ephrin-B2 and the anatomical location of the thymus. E18.5 mutant *ephrin-B2*$^{\Delta V/\Delta V}$ (left), mutant *ephrin-B2*$^{5Y/5Y}$ (middle) and control *ephrin-B2*$^{+/+}$ (right) embryos were dissected to reveal the thymus in the thoracic cavity. Black arrows; thymus.

Figure 6.5. NC derived cells deficient in ephrin-B2 exhibit abnormal polarisation and migration. (A-D) Cells from E13.5 control *Wnt1-Cre;Rosa26*^{eYfp} or mutant *Ephrin-B2*$^{Lx/Lx}$*;Wnt1-Cre;Rosa26*^{eYfp} thymi were incubated with or without ephrin-B2/Fc cross-linked with IgG or EphB4/Fc for 2 hours in serum-free IMDM, as indicated on the figure, and imaged by fluorescence microscopy. Images of cells are shown at 0, 2 and 4 hour time points. All images are 60x magnification. **(E)** Total displacement of the cells shown in figures A-D. T-test; A-B p=0.000315, A-C p=0.000374, B-D p=0.0228. **(F)** Meandering index was calculated for each cell shown in A-D. 1 indicates directional movement, 0 indicates random movement. T-test; A-B p=0.00101, A-C p=0.00134, B-D p=0.0769. Similar results were obtained from at least 3 experiments for each condition.

Figure 6.6. Whole organ culture of wildtype and ectopic thymic rudiments. Whole thymic rudiments from E13.5 control *Wnt1-Cre;Rosa26*^{eYfp} (left) and mutant *Ephrin-B2*$^{Lx/Lx}$*;Wnt1-Cre;Rosa26*^{eYfp} (right) embryos were dissected and embedded in a collagen gel. The gels were incubated for 48 hours, fixed with paraformaldehyde and stained with an antibody detecting eYFP. The thymi were imaged by confocal microscopy and are presented as single confocal sections (top) or a three dimensional rendering (bottom). Red arrow indicates a small fragment of thymic tissue that appears to have been dragged away.


A
E12.5
Upper cervical region
Lower cervical region
wild type
mutant
*
th
pt
th
th
B
E13.5
Cervical region
Thoracic region
wild type
mutant
*
*
th
th
*
*


E12.5
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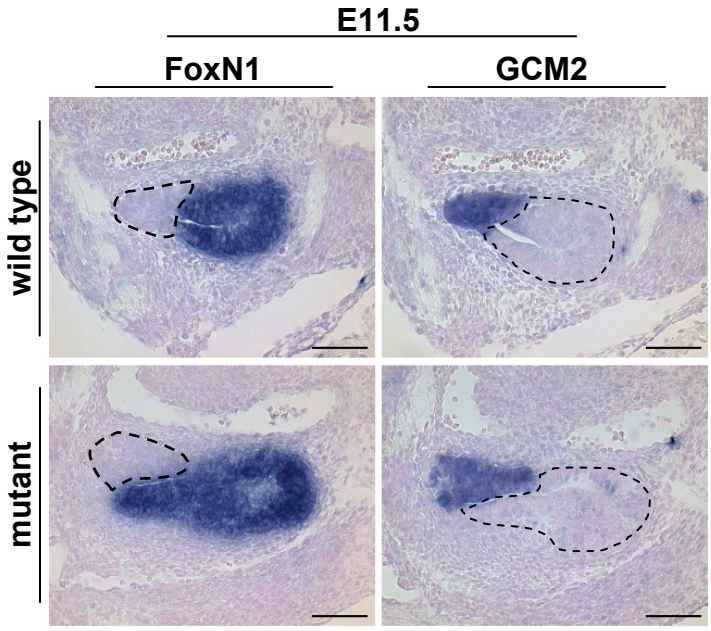
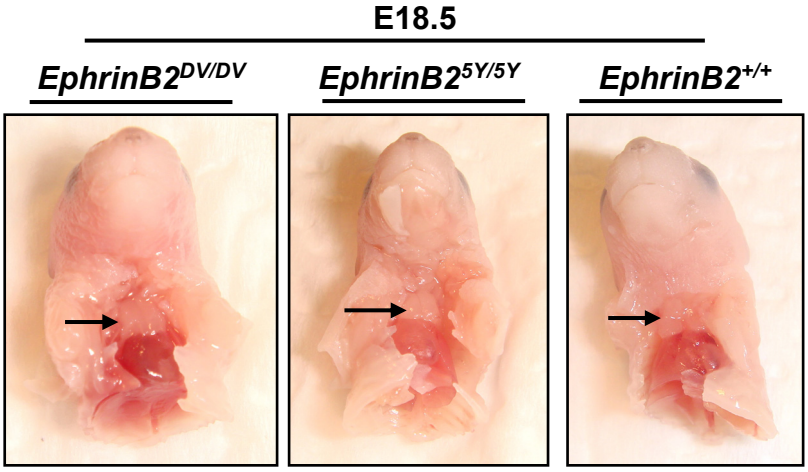
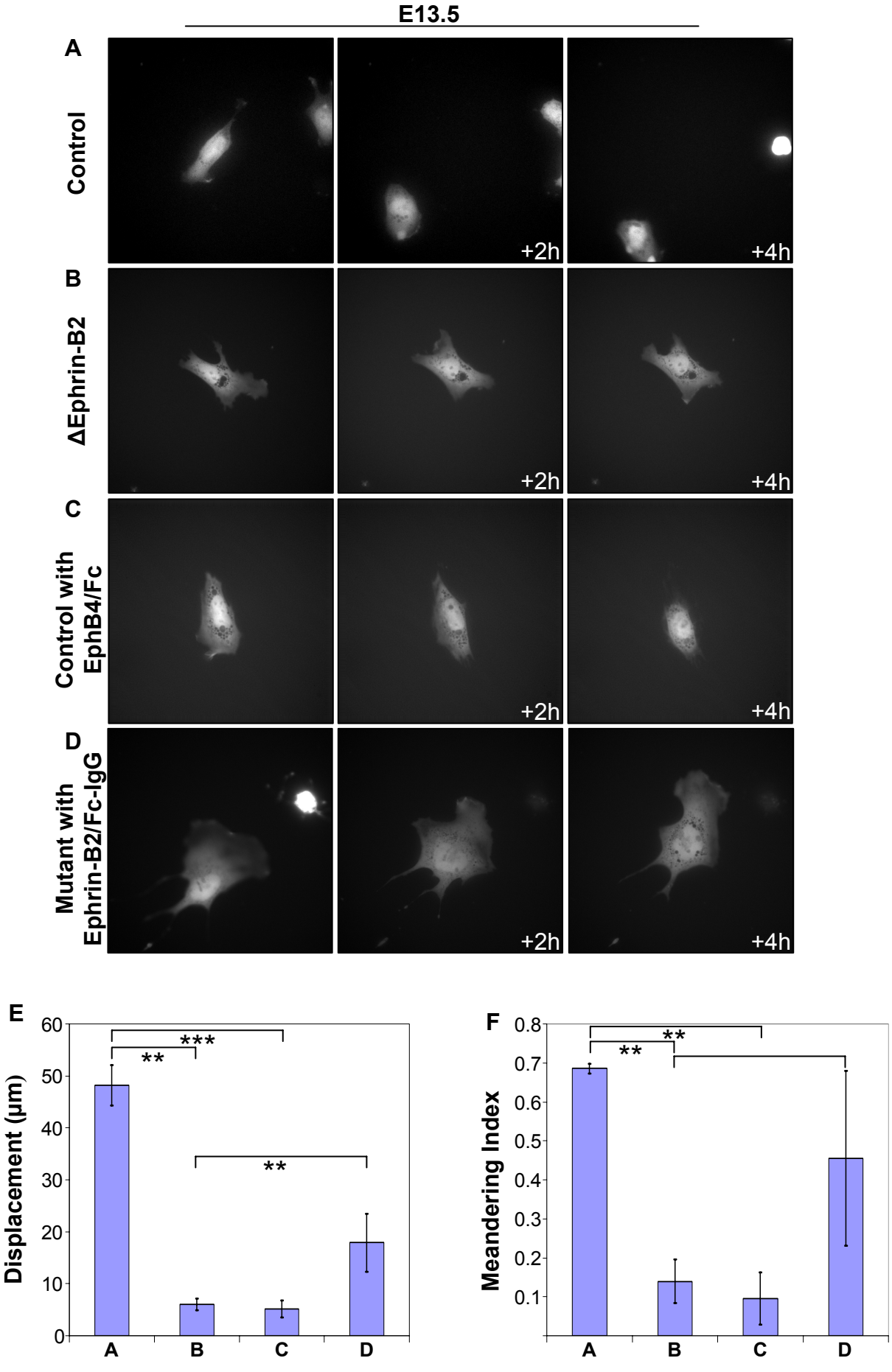
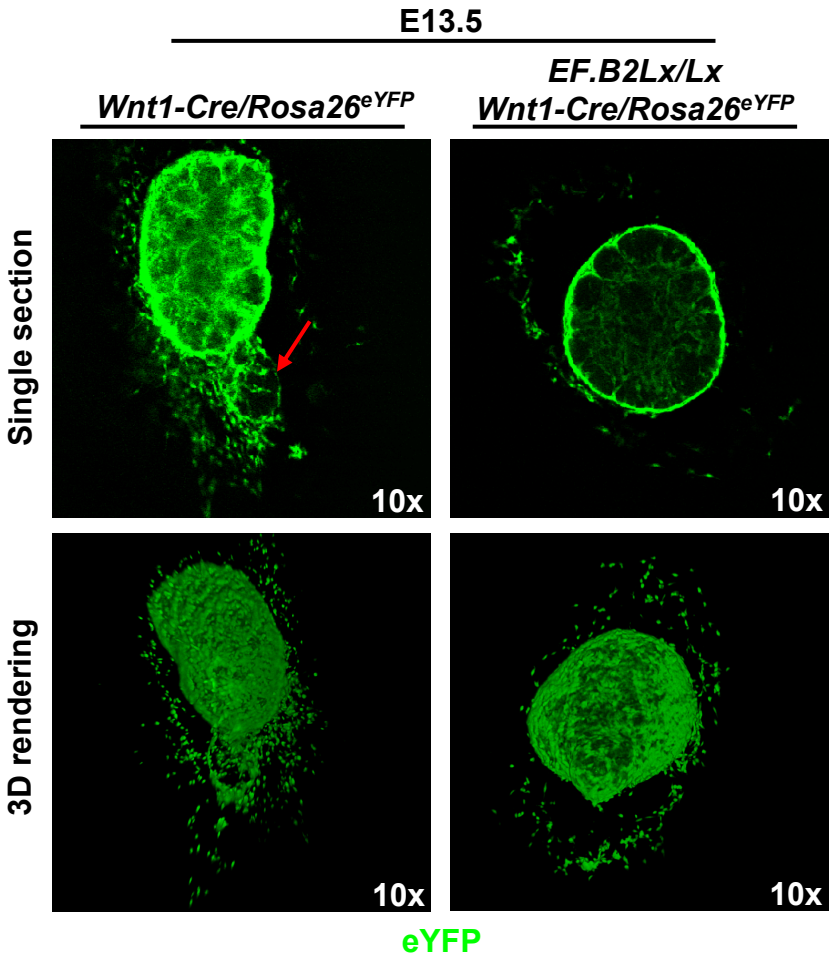

E11.5
FoxN1
GCM2
wild type
mutant


E18.5
EphrinB2DV/DV
EphrinB25Y/5Y
EphrinB2+/+


E13.5
A
Control
+2h
+4h
B
ΔEphrin-B2
+2h
+4h
C
Control with EphB4/Fc
+2h
+4h
D
Mutant with Ephrin-B2/Fc-IgG
+2h
+4h
E
Displacement (μm)
60
50
40
30
20
10
0
***
**
**
A
B
C
D
F
Meandering Index
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
**
**
A
B
C
D


E13.5
Wnt1-Cre/Rosa26eYFP
EF.B2Lx/Lx
Wnt1-Cre/Rosa26eYFP
Single section
3D rendering
10x
10x
10x
10x
eYFP

Chapter Seven: General Discussion

How does NC derived mesenchyme influence the migration of the thymus into the thoracic cavity?

The data presented in this thesis implicate Eph/ephrin expression by NCCs in the positioning of the thymic rudiment. However the mechanism by which this occurs is unclear. It is possible that NCCs surrounding and within the organ have an intrinsic ability to direct the migration of the organ. Equally, molecules expressed by cells surrounding the thymus could be involved in attracting or repelling the rudiment, or, a combination of intrinsic and extrinsic signals could be responsible for organ migration.

NC derived cells exit the dorsal neural tube and migrate extensively, colonising almost all tissues of the embryo. Individual NCCs migrate with high persistence towards their targets (188), but the mechanism that controls directionality are unknown. A number of molecules are known to have a role in NC migration, including ephrins, semaphorins, *Roundabout* (Robo) and its ligand Slit, and Glial cell-line Derived Neurotrophic Factor (GDNF), but the majority appear to act by repulsing the NCCs into specific domains. So far, no chemoattractant molecules have been described for the NC. Instead, regulation of the cell's cytoskeleton through GTPases and, thus, its polarity are proposed to regulate, at least partly, directional NC migration. It was recently shown that Syndecan 4 inhibition of Rac activity and planar cell polarity (PCP) signalling promotes RhoA activity. These molecules regulate

cell polarity and the cytoskeletal machinery that controls the formation of cell protrusions (273).

Since Eph/ephrins regulate members of the Rho and Ras families of GTPases, including Rac, it is possible that deficiencies in Eph signalling in the mutants described here causes a loss in cell polarity and inhibits changes in cytoskeletal structure, which renders the cell unable to migrate. In order to investigate this possibility it will be necessary to determine whether polarity or the cytoskeleton is disrupted in the ephrin-B2 deficient NCCs. There are a number of ways to do this including imaging molecules involved in polarity, such as Cdc42, Par3, Par6 and aPKC in the mutant cells. Additionally, it will be necessary to establish whether molecules downstream of the Eph receptor are downregulated in the ephrin-B2 deficient NCCs. To do this, it may be possible to compare the expression of genes involved in cell polarity and regulation of the cytoskeleton from microarray data obtained from wild type NCCs and those deficient for ephrin-B2. Moreover, interfering with the putative signalling pathways downstream of Eph receptors, by blocking Rac, Cdc42 or the polarity proteins of cells *in vitro* may indicate how Eph/ephrin signalling controls migration. This could be done using a similar method to that used here, where individual NC derived cells from control or mutant embryos are cultured in the presence of molecules that inhibit Rac, Cdc42 or polarity proteins. If interfering with these proteins in wild type NCCs produces a similar phenotype to the mutant cells,

this would indicate that they could be members of the same signalling pathway as Eph receptors. Likewise, it may be possible to change the phenotype of the mutant NCCs back to polarised and migratory wild type NCCs by stimulating molecules that are thought to be downstream of EphB receptors.

In addition to intrinsic signals from NCCs, the environment that surrounds the thymic rudiment may also play an important role in thymus migration. There are many possible candidates for this function, including the FGF family of proteins. FGFs play a crucial role in embryogenesis, including the collective cell migration of the lateral line in zebrafish (272), and NCC migration (274). Interestingly, FRS2a mutant mice that have a targeted disruption in their binding site also exhibit ectopic thymi (26). It is tempting to suggest that the ectopic location of thymi in these mice is due to a defect in migration, but it was demonstrated that incomplete separation was actually the cause of the thymus location defect. Overexpression of FGFs also results in ectopic organs. *Sprouty* mutants, which are deficient in inhibiting FGF signalling, also have ectopic thymi and general separation defects from the pharynx (250). Thus, a tight balance of FGF signalling is important for thymus development, however, it is not clear how these signals function and whether they are involved in the ectopic thymus in the mutants described here.

Semaphorins (Sema) are secreted or membrane-bound proteins and may be attractive candidates in determining the migratory path of the thymus. Members of the semaphorin family were originally characterised as repulsive guidance cues for neuronal precursors in the nervous system. Semaphorins are also implicated in the segregation of NCCs into migration streams in the hindbrain of in the chick and zebrafish (275, 276). Recently, it was demonstrated that they also may have a role in organogenesis in other tissues, for instance, the heart. Mice deficient for Sema 3A have heart defects resulting from abnormalities during morphogenesis (277, 278).

Sema 3D and Sema 3F are expressed specifically in the developing parathyroid and thymus. Receptors for Class 3 Semaphorins, including Neuropilin-1 and Neuropilin-2, are detected on the arterial vessel walls and peripheral nerves adjacent to the third pharyngeal pouch (279). Moreover, expression of Neuropilin-1 and -2 were detected at E12.5, just before the thymus and parathyroid glands migrate, on the cells that surround both organs. These data suggest that interactions between Semaphorins expressed on cells associated with the thymus and parathyroid, and their receptors including Neuropillins on the mesenchyme, vessels and nerves around the third pouch, could have a role in organ migration. Signalling downstream of semaphorins is unclear, but may regulate the cytoskeleton and the organisation of actin filaments and the microtubule network.

It is possible that the thymic rudiment follows arteries, which arise in the pharyngeal arches, down into the thoracic cavity where they form the major vessels of the heart, similar to the proposed mechanism of thyroid development. Like the thymus, the thyroid derives from the pharyngeal pouch endoderm and separates by E11.5. By E13.5, it has relocated to its final resting position in the midline at the ventral side of the trachea. It subsequently bifurcates to form two lobes that develop in close contact with the carotid arteries, which appear to determine its location. Alt and colleagues (280) recently demonstrated that ectopic vascular cells are sufficient to mislocate thyroid tissue in a non-cell autonomous manner. Since the thyroid is formed in close proximity to the carotid arteries, these authors concluded that vessels may determine the position of the thyroid. It is possible that vessels also direct the relocation of the thymus from the pharyngeal pouches to the thoracic cavity in a similar mechanism. In support of this hypothesis are the data presented here indicating that Eph and ephrins are expressed on the NC derived mesenchymal cells present around and within the rudiment, and that in the absence of ephrin-B2 the location of the organ is ectopic. Thus, interactions with reciprocal Eph receptors for ephrin-B2 expressed on the vessels that form nearby in the pharyngeal arches could direct the migration of the rudiment towards the heart.

Although both thymus and thyroid partly derive from the NC, there are differences between the development and function of the two organs. In

contrast to the thymus, the function of the thyroid is compromised in an ectopic location, resulting in congenital hypothyroidism (281). However, since the NC contributes mesenchyme to both organs and they undergo similar relocalisation, it will be important to determine whether the blood vasculature plays a role in thymus positioning. This could be done in a similar way to experiments that showed that thyroid tissue was attracted by ectopic vascular cells. In these experiments, cells which have been forced to adopt an endothelial fate by injection of Scl and Lmo2, transcription factors specifically required for haematopoiesis and angiogenesis (282), were injected into wild-type hosts. This created a mosaic of wild-type and ectopic endothelial cells. In these experiments, tissue from the thyroid projected towards the grafted cells. It may be possible to use the same technique to determine whether cells from the thymus are attracted to blood vessels. Alternatively, culturing endothelial cells and thymic rudiments in a three dimensional collagen gel may produce similar results.

Interestingly, PDGFRα was also recently implicated in NCC migration. In the absence or knock-down of PDGFRα using a hypomorphic allele or antisense morpholinos, NCCs failed to migrate rostrally and ventrally around the eye when compared to wild type control embryos. When the ligand, PDGF-AA, was overexpressed by injecting a *pdgfaa* morpholino in zebrafish, NCCs bunched at the optic stalk and failed to continue their migration to the palatal precursor (283). These authors suggest that this is the cause of the facial

and palatal cleft defects in the PDGFRα mutant mice. It was further suggested that a micro RNA, Mirn140, binds to the 3' end UTR of the PDGFRα mRNA and interferes with its translation (283). Thus, PDGFRα first attracts NCCs then its expression is reduced allowing the NCCs to continue along their normal pathway of migration. In chapter four it was shown that PDGFRα is downregulated after E15.5 in the NCCs that contribute to the thymic rudiment, and this coincides with the thymus reaching its destination. It is possible that interactions between PDGFRα expressed by the NCCs and PDGF-AA ligand, expressed by surrounding tissues may be the attractant for thymus migration. In order to examine this possibility, expression levels of PDGFRα should be determined in wild type NCCs and in those deficient in ephrin-B2. In addition, ephrin-B2 expression on NCCs should be measured in mice lacking the PDGFRα receptor.

The signalling molecules and transcription factors mentioned here are by no means an exhaustive review of all the molecules potentially involved in NCC, or thymus, migration. Clearly the mechanism of NCC migration is complex and may be regulated through a delicate equilibrium of attractant and repellent signals, and intrinsic and environmental signals. Eph/ephrin signals may be chemokinetic, thus, they stimulate the migration of NCCs but with no directionality. Subsequently, directionality is determined by signals extrinsic to the NCC, which remain to be defined. Investigations into these signals should include members of the Hox, Pax, BMP, SHH and FGF families of

genes, since mice with mutations in these genes also have thymus abnormalities including ectopia. To do this, expression of ephrin-B2 or its preferred receptor, EphB4, should be examined in other mutants with ectopic thymi. Likewise, genes including the Hox, Pax, BMP, SHH and FGF families should be examined during thymus development in the mutants with ephrin-B2 deficient NCCs.

Summary

The aim of this thesis was to analyse the role of NC derived cells in thymus organogenesis. Previously published reports indicated that NC derived cells are crucial in the development of the thymic rudiment through their interactions with endoderm derived TECs. However, once vascularisation of the rudiment occurs, and TECs are competent in their ability to support T cell development, NCCs were thought to be lost or replaced by cells of other mesenchymal origin. Using the NC specific *Wnt1-Cre* and *Sox10-Cre* transgenes to drive expression of eYFP from the ubiquitously expressed Rosa26 locus, NC derived cells were observed in the newborn and adult thymus. Similar to an earlier report in 1975, which showed that NC derived cells formed the connective tissues surrounding the blood vessel in the thymus of chick-quail chimeras (13), it was demonstrated here that NC derived cells differentiate into perivascular mesenchyme.

The processes of patterning of the third pharyngeal pouch into thymus and parathyroid fated domains, and their subsequent separation from each other and the pharynx, have been described elsewhere. However, mechanisms of thymus relocation into the thoracic cavity are unknown. In order to dissect the roles of NCCs and TECs in these processes ephrin-B2, a member of a family of tyrosine kinase receptors and their ligands that are required for processes including cell sorting, boundary formation, adhesion and migration elsewhere in the developing embryo, was ablated. ephrin-B2 ablation from TECs and NCCs does not appear to result in an overt immune phenotype. In contrast, ephrin-B2 expression on NCCs was required for the normal relocation of the thymic rudiment into the thoracic cavity. Other mutant mice have been described with ectopically located thymus organs in the cervical region, however, all exhibited defects in initial patterning or separation of the rudiment from the pharynx and/or parathyroid gland. Evidence is presented that indicates initial patterning of the thymus/parathyroid combined rudiment is normal in the mutant mice described here, and that separation from the pharyngeal pouch is likewise not affected. In contrast, separation of the combined thymus/parathyroid domains is delayed in the ephrin-B2 deficient NCC mice. Thus, there are two possible explanations for the ectopic location of the thymic rudiment in the mutant mice described in this report. Delayed separation of the thymus and parathyroid primordia may hold the thymic rudiment in the cervical region whilst the physical pathway of migration into the thoracic cavity no longer permits relocation of the rudiment. Equally

possible, the process of migration of the thymus may be impaired in the absence of ephrin-B2 expression on NCCs. In order to distinguish between these two possibilities, the migration of individual NC derived cells was examined. NC derived cells deficient in ephrin-B2 exhibited defects in polarisation and, thus, were unable to migrate. Thus, it is proposed that the migration of individual NC derived cells directs the relocation of the thymic rudiment by a process of collective cell migration. To our knowledge, this is the first report that implicates Eph/ephrin signalling on NC derived cells in the relocation of the thymus, which appears to occur by a process of collective cell migration. Collective cell migration occurs in a number of processes such as morphogenesis, tissue repair and cancer invasion and metastasis. Thus, understanding the molecular mechanisms which bring about the collective migration of the thymic rudiment are of interest to a wide range of biologists.

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